

Multiple roles for Nav1.9 in visceral afferent activation by noxious mechanical and inflammatory stimuli

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ABSTRACT

Chronic visceral pain affects millions of individuals worldwide, remains poorly understood, and current therapeutics are constrained by undesirable adverse events. Inflammation and distension of visceral organs are common causes of pain, suggesting drugs targeting these signalling pathways may be efficacious visceral treatments. The voltage-gated sodium channel subtype 1.9 (Nav1.9) has been strongly associated with the development of inflammatory pain by rodent studies and more recently, by the identification of channelopathies in man. The aim of these studies was to investigate the role of Nav1.9 in visceral afferent signalling in the gut. Data from this thesis demonstrates that Nav1.9 is expressed by approximately half of gut-projecting rodent dorsal root ganglia sensory neurons. Consistent with significant expression in visceral afferents, Nav1.9 is required for normal mechanosensation, and for the direct excitation and mechanical hypersensitisation of mouse colonic afferents by inflammatory mediators applied as an inflammatory soup (bradykinin, ATP, histamine, PGE₂, and 5HT) or derived from man (as inflammatory bowel disease tissue supernatants). Additionally, the importance of P2Y receptor activation in both rodent and human gut to algogenic purinergic signalling was demonstrated. Collectively, these results demonstrate that Nav1.9, is required for persistence of responses to intense mechanical stimulation, contributes to inflammatory mechanical hypersensitivity, and is essential for activation by noxious inflammatory mediators, including those from diseased human bowel. These findings suggest that Nav1.9 represents a high-value target for development of visceral analgesics.

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PRESENTATIONS AND AWARDS

Some results contained within this thesis have been published previously and presented at both national and international scientific meetings.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
AC	adenylyl cyclase
ADP	adenosine-5'-diphosphate
AP	action potential
ASIC	acid sensing ion channel
ATP	adenosine-5'-triphosphate
BDNF	brain derived neurotropic factor
BK	bradykinin
cAMP	cyclic adenosine-5'-monophosphate
Cap	capsaicin
Cav	voltage-gated calcium channel
CCI	chronic constriction injury
CCK	cholecystokinin
CD	Crohn's disease
CFA	complete Freud's adjuvant
CG	celiac ganglion
CGRP	calcitonin gene-related product
CMMC	colonic migrating motor complexes
CNS	central nervous system
COX	cyclooxygenase
CRD	colorectal distension
CV	conduction velocity
DEG/ENaC	degerin/epithelial Na ⁺ channel
DEPC	diethylpyrocarbonate

DRG	dorsal root ganglion
DTT	dithiothreitol
ENS	enteric nervous system
ERK	extracellular signal-regulated kinase
FB	fast blue
FRAP	fluoride-resistant acid phosphatase
GABA	γ -aminobutyric acid
GDNF	glial cell line-derived neurotrophic factor
GFR α	GDNF receptor α
GI	gastrointestinal
GLP1	glucagon-like peptide-1
GM-CSF	granulocyte macrophage colony-stimulating factor
GPCR	G-protein coupled receptor
GTP γ S	guanosine-5'-O-(3-thiotriphosphate)
HCN	hyperpolarisation-activated cyclic nucleotide gated channel
HRP	horseradish peroxidase
HT	high threshold
IB4	<i>Griffonia simplicifolia</i> isolectin B4
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IGLE	intraganglionic laminar ending
IHC	immunohistochemistry
IL	interleukin
IMA	intramuscular array
IMG	inferior mesenteric ganglion
IMN	intermesenteric nerve

IPAN	intrinsic primary afferent neurone
IR	immunoreactivity
IS	inflammatory soup
ISH	<i>in situ</i> hybridisation
K _{Ca}	calcium-activated potassium channel
K _{ir}	inwardly rectifying potassium channel
K _v	voltage-gated potassium channel
LSN	lumbar splanchnic nerve
LT	low threshold
NACC	National Association for Colitis and Crohn's Disease
Nav	voltage-gated sodium channel
NF200	neurofilament (heavy)
NG	nodose ganglia
NGF	nerve growth factor
NSAID	non-steroidal anti-inflammatory drug
P2X	ionotropic P2X purinoceptor
P2Y	metabotropic P2Y purinoceptor
PAR	protease-activated receptor
PBS	phosphate buffered saline
PFA	paraformaldehyde
PG	pelvic ganglion
PGE ₂	prostaglandin E ₂
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PNS	peripheral nervous system

PVG	pre-vertebral ganglia
rIGLE	rectal intraganglionic laminar ending
RMP	resting membrane potential
RT-PCR	real-time polymerase chain reaction
SMG	superior mesenteric ganglion
SNRI	serotonin-norepinephrine reuptake inhibitor
SP	substance P
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressant
TG	trigeminal ganglia
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF	tumour necrosis factor
trkA	high-affinity nerve growth factor receptor
TRP	transient receptor potential channel
TTX-S/R	tetrodotoxin-sensitive/resistant
UC	ulcerative colitis
UDP	uridine-5'-diphosphate
UTP	uridine-5'-triphosphate
VIP	vasoactive intestinal polypeptide
VMR	visceromotor reflex
WDR	wide dynamic range

CHAPTER 1: INTRODUCTION

1.1 CLINICAL IMPACT OF VISCERAL PAIN IN INFLAMMATORY BOWEL DISEASE

Chronic visceral pain affects millions of individuals worldwide, is a leading reason for presentation to a surgeon or gastroenterologist (Bhuiya, Pitts, & McCaig, 2010; Bonica, 1990) and is often the first indication of more serious disease, including appendicitis, cancer, ischaemia and gastritis. One group of patients where visceral pain incurs distinct morbidity is in those suffering from inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC). This disease, which affects approximately 240,000 in the UK, is a chronic and relapsing inflammatory condition caused by a hyperinflammatory T-cell response to certain commensal enteric bacteria (Sartor, 2006). The disease presents clinically as diarrhoea, bleeding, fatigue and abdominal pain (Mowat et al., 2011). Abdominal pain is rated highly as a negative impact on patient's lives (R. Driscoll, 2008; Ghosh & Mitchell, 2007), and is more strongly associated with Crohn's disease than ulcerative colitis (Aghazadeh et al., 2005; Castro et al., 2008; Ghosh & Mitchell, 2007; Kim et al., 2010; Singh et al., 2011; Y. Wang, Ouyang, & group, 2007). A survey conducted by the National Association for Colitis and Crohn's Disease (NACC) of 2527 of its members, found strong associations with the intensity of pain experienced and disease activity (R. Driscoll, 2008). Indeed, 72% of patients described the pain as an indication that a 'flare-up' was occurring. As such, a significant proportion of IBD patients experience visceral allodynia to physiological bowel movements and inflammatory hyperalgesia is associated with the vast majority of patients. The pain experienced is not limited to active disease or 'flare-ups', with up to half of

patients also reporting abdominal pain during remissive states (Bielefeldt, Davis, & Binion, 2009; R. Driscoll, 2008; Singh et al., 2011). Furthermore, continued pain during disease acquiescence indicates long-term mechanistic changes to nociceptive sensory pathways, resulting in visceral hypersensitivity. The cause of pain in IBD is poorly understood, however chronic serosal and mucosal inflammation is likely to result in peripheral nerve sensitisation, resulting in visceral hypersensitivity to physiological gastrointestinal (GI) function and in some patients, secondary irritable bowel syndrome (Grover, Herfarth, & Drossman, 2009; Knowles & Aziz, 2009). Central sensitisation and psychological factors will also significantly impact pain thresholds (Mowat et al., 2011). These factors are perpetuated by distension and obstruction caused by occlusions and strictures as a result of the disease (Bielefeldt et al., 2009).

With almost a third of patients having been admitted to hospital at least twice because of pain (R. Driscoll, 2008), current medical practices are not able to effectively provide management of abdominal pain in these patients. Given the complex factors affecting abdominal pain in IBD, the treatments used to manage pain are equally diverse, including pharmacological, behavioural and procedural methods. In early stage disease, the use of standard of care IBD treatments (e.g. corticosteroids, aminosalicylates, antibiotics, enteral therapy, immunomodulators and biological response modifiers) can reduce intestinal inflammation and drive a large subset of patients into remission. This may also reduce associated abdominal pain, but as mentioned previously, continued visceral hypersensitivity, discomfort and pain can persist in up to half of patients. As such, it is likely that there exists a subset of patients in which

abdominal pain is discontinuous to inflammation and disease activity.

According to the NACC patient survey, the most commonly prescribed medicines specifically for pain relief in IBD were paracetamol (44%), ibuprofen (24%), anti-spasmodics (24%) and tramadol (11%) (R. Driscoll, 2008). These medicines represent the major classes of pharmacological interventions in IBD pain management; however each drug category is constrained in both effectiveness and use by associated negative adverse effects (see Table 1).

Common non-steroidal anti-inflammatory drugs (NSAIDs), whilst effective at reducing abdominal pain and IBD-related musculoskeletal symptoms, have been linked with an exacerbation of disease activity and an increased likelihood of flares and relapse (Bonner, Walczak, Kitchen, & Bayona, 2000; Evans, McMahon, Murray, McDevitt, & MacDonald, 1997; Kefalakes, Stylianides, Amanakis, & Kolios, 2009; Mowat et al., 2011). Indeed, increased concern about the correlation between NSAIDs and IBD exacerbation led to the use of selective cyclooxygenase-2 (COX-2) inhibitors (including celecoxib, rofecoxib and etoricoxib) in IBD, which lack COX-1 activity, the proposed mechanism for inducing disease activity. The effectiveness of this class of drug remains controversial and, as yet, has failed to demonstrate robust anti-inflammatory effects and have further been associated with a 7-16% increase in disease exacerbation (Biancone, Tosti, De Nigris, Fantini, & Pallone, 2003; Mahadevan, Loftus, Tremaine, & Sandborn, 2002). In irritable bowel syndrome (IBS), anti-spasmodics (such as hyoscyamine) have proven effective in the management of abdominal pain and may be used to reduce pain evoked by intestinal inflammation or partial obstruction (Ford et al., 2008). No equivalent evidence exists for their usage in IBD (Makharia, 2011), although anti-spasmodics are

commonly used when remission is induced in IBD patients. However, side effects must be monitored, with complete obstruction and worsening of gut dysmotility both possible outcomes. The chronic use of narcotics also has negative impacts on morbidity and mortality due to their effects on gastrointestinal function and dysmotility in IBD patients, alongside established concerns around abuse (Cross, Wilson, & Binion, 2005; Edwards, Radford-Smith, & Florin, 2001; Mowat et al., 2011). Reported use of narcotics is as high as 70% in hospitalised IBD patients, with between 5-13% of patients prescribed chronic narcotics as outpatients (Edwards et al., 2001; Long, Barnes, Herfarth, & Drossman, 2012). As such, the adverse effects associated with different analgesic classes significantly limits the therapeutic options available for treating visceral pain in IBD.

Other medications that are used in the management of visceral pain in IBD and associated visceral hypersensitivity include psychotropic agents. The use of these medications stems out of current IBS literature, with the targeting of serotonin and noradrenaline purported to modulate visceral motility and sensation (Grover & Drossman, 2011). Whether benefits arise from direct effects on nociception or from psychological improvements to depression and anxiety, which are often associated with chronic abdominal pain, remains an unknown. The use of low-dose tricyclic antidepressants has proven to be effective in alleviating abdominal pain in IBS, and qualitative evidence exists for their effectiveness in IBD (Mikocka-Walus et al., 2007; Rahimi, Nikfar, Rezaie, & Abdollahi, 2009). Studies investigating the use of selective serotonin-reuptake inhibitors (SSRIs) in both IBS and IBD are less convincing, but suggest that

SSRIs may have utility in the treatment of functional abdominal pain associated with IBD (Walker, Gelfand, Gelfand, Creed, & Katon, 1996). Finally, the use of centrally-acting gabapentin and pregabalin, which show efficacy in the treatment of neuropathic pain, also likely have relevance in the treatment of centrally and inflammatory-mediated visceral hypersensitivity (Gale & Houghton, 2011; Srinath, Walter, Newara, & Szigethy, 2012). In addition, procedural approaches have benefit in some patients, including acupuncture, nerve blocks and transcutaneous electrical nerve stimulation (Srinath et al., 2012). Classical psychological pain management aimed at modulating central pathways influencing sensory processing is also applicable to patients with IBD and may include cognitive behavioural therapy, hypnotherapy and stress management (Srinath et al., 2012).

Given that chronic visceral pain is a debilitating and poorly treated component of IBD, there is a clear need for novel pain therapies to provide benefit to those suffering from these conditions (R. Driscoll, 2008).

Drug class	Example drugs	Mechanism of action	Role in IBD pain management	Possible complications/ limitations
<i>Pharmacological</i>				
Anti-spasmodics	Hyoscyamine Dicyclomine	Nonselective inhibitor of acetylcholine receptors	Smooth muscle relaxant, reducing intestinal spasm associated with inflammation or partial obstruction	Fulfilment obstruction/ worsening of gut dysmotility
NSAIDs	Ibuprofen Diclofenac	Nonselective inhibitor of COX enzymes	Provides analgesic and anti-inflammatory effects in GI tract and associated musculoskeletal pain	May exacerbate IBD activity, induce relapse and increase frequency of flares
COX-2 inhibitors	Celecoxib Etoricoxib Rofecoxib	Selective inhibitor of COX-2 enzyme	Limited; purported to provide analgesic and anti-inflammatory effects	May exacerbate IBD activity
Narcotics	Morphine Tramadol Hydromorphone	Modulators of opioid receptors	Useful for post-operative and severe chronic pain	Nausea, vomiting, constipation, intestinal pseudo-obstruction and reduced GI motility, alongside addiction and substance abuse
<i>Psychotropic</i>				
TCAs	Amitriptyline Desipramine Dosulepin	Nonselective SNRIs and inhibitors of 5-HT receptors, amongst others	May decrease comorbidities of anxiety and depression; may affect nociception by modulating visceral motility and sensation	Inconclusive supporting evidence; may be ineffective for non-depressed patients
SSRIs	Paroxetine Citalopram	Inhibitors of SERT	Similar proposed mechanism to TCAs	Inconclusive supporting evidence; may be ineffective for non-depressed patients
SNRIs	Duloxetine Venlafaxine	Inhibitors of NET	Similar proposed mechanism to TCAs	Inconclusive supporting evidence; may be ineffective for non-depressed patients
Gabapentin/ Pregabalin	Gabapentin Pregabalin	Modulators of voltage-gated calcium channels	Centrally acting analgesic	Side effects are mild; no extensive supporting evidence in IBD pain

Table 1. Pharmacological treatment of IBD pain. COX, cyclooxygenase; NET, norepinephrine transporter; NSAIDs, non-steroidal anti-inflammatory drugs; SERT, serotonin transporter; SNRI, selective norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressants.

1.2 CHARACTERISTICS OF VISCERAL PAIN

Visceral pain is a sensation that forms part of the body's defence strategy against harmful stimuli, specifically those affecting hollow organs. Noxious stimuli are transduced by primary afferents via spinal pathways to the parts of the brain responsible for integrating different aspects of the sensory experience, including discrimination, emotion and cognition. In IBD, visceral pain is associated with active disease, and with visceral hypersensitivity driven by sensitising processes in visceral pain pathways. As such, chronic visceral pain in IBD may be considered a function of inflammatory and neuropathic pain resulting from GI peripheral nerve injury. Ongoing primary afferent input is likely to be critical for maintaining such chronic pain sensations (Haroutounian et al., 2014). Therefore, the processing of visceral nociception and transduction of noxious stimuli by visceral sensory receptors will be covered in more detail, specifically focusing on visceral afferent signalling and processing relevant to chronic visceral pain associated with IBD.

Visceral afferent fibres transduce conscious sensations and act to regulate autonomic function from the vast majority of mammalian visceral organs. The activation of visceral afferents resulting in reflex actions occurs mainly at the subconscious level, with some solid organs such as the lungs or liver being insensitive to pain. Indeed, the activation of pulmonary stretch receptors or hepatic chemoreceptors is not consciously perceived (Cervero, 1985), and as such considerable damage may occur before detection. By contrast, in hollow viscera relatively mild inflammation or tonic contraction of smooth muscle can cause intense pain. Whilst it is not possible to withdraw from such stimuli as

you would with the rapid movement of a limb from noxious somatic stimuli, protective mechanisms in the form of long-term behavioural coping strategies are often evoked by such pain (e.g. nausea, changes to water and food intake, and general inactivity). Within the gastrointestinal tract, in conjunction with autonomic regulation (absorption, secretion and propulsion), afferent fibres contribute to conscious sensations such as fullness, bloating, urgency, and following noxious stimulation, pain (Knowles & Aziz, 2009). These sensations are the combination of innervation originating from intrinsic sensory neurones, pre-vertebral ganglia projecting viscerofugal fibres, and both spinal and vagal extrinsic afferents (Grundy, 2002). Visceral afferents of the gastrointestinal tract may encode stimuli ranges in the innocuous or noxious domains, as well as intensity encoding across the full intensity range. This is demonstrated where innocuous rectal sensation may rapidly evolve into pain and discomfort if maintained (Cervero, 1988). The sensory modalities familiar to the skin of touch, wetness, prickle, temperature and pain are mediated by specific receptors for mechanosensation, chemosensation and thermosensation, which differ both anatomically and molecularly from those associated with visceral sensation. Indeed, pain is the only sensation that may be evoked by the majority of visceral organs. In conscious man, stimulation by balloon distension can evoke pain throughout the GI tract, whilst other stimuli such as cutting, pinching, local stretching and electrical stimulation all fail to elicit pain (Bentley & Smithwick, 1940; Ray & Neill, 1947; "The Goulstonian Lectures ON THE SENSIBILITY OF THE ALIMENTARY CANAL IN HEALTH AND DISEASE," 1911). This is consistent with the stimulation of bowel mesentery causing painful sensation and the inability to perceive temperature in the lower GI tract (Ray &

Neill, 1947). Whilst the GI tract is extensively innervated, the sensation of pain is primarily transduced by spinal afferents, as shown by its absence following bilateral sympathectomy (Ray & Neill, 1947). These studies highlight specialisation in visceral afferent signalling to noxious stimuli and processing that are unique to the viscera.

Visceral sensation and pain is poorly graduated and localised, with no specific pathway present within the central nervous system (CNS) processing visceral nociceptive signals (G. F. E. Gebhart, 1995). Visceral unmyelinated afferent neurones project centrally over multiple spinal segments and diffusely across the dorsal horn. This is in contrast to cutaneous unmyelinated afferent neurones, which are much more restricted in their central arborisations (Jänig, 1996). Visceral afferent fibres may also possess multiple peripheral receptive fields (Berthoud, Lynn, & Blackshaw, 2001). As such, the low density of visceral afferent innervation, in conjunction with the non-existent organotopic representation and broad segmental projection on the dorsal horn, all contribute to the generalisation and poor graduation of visceral sensation (Jänig, 1996). The poor definition of visceral pain in the CNS leads to the manifestation of pain in unaffected areas of the body, including left lower quadrant, neck and left arm, dependent on the visceral organ affected (Sikandar & Dickenson, 2012). This is also contributed to by viscera-somatic convergence in ascending afferent pathways (Cervero, Laird, & Pozo, 1992), with patients experiencing referred visceral pain in associated somatic structures. Visceral pain may also evoke involuntary non-specific motor responses and is strongly associated with autonomic reflexes, including symptoms such as sweating,

nausea, changes in body temperature and heart rate (Knowles & Aziz, 2009). Finally, there exists a strong affective component to visceral pain, which may be reinforced by psychological conditions such as depression or anxiety (Knowles & Aziz, 2009). Collectively, the divergent characteristics of visceral pain and the activation of associated CNS and autonomic pathways present challenges for the identification of visceral nociceptors, when compared to the more discrete pain pathways present for somatic systems (Cervero & Laird, 2004). However, by investigating the specific anatomical and molecular pathways mediating the transduction of visceral pain in conditions such as IBD, it will be possible to identify novel mechanisms relevant to visceral nociception and inflammatory pain. Specifically, Crohn's disease primarily presents as an ileocecal condition whilst ulcerative colitis is typically localised more distally; both activating the spinal afferent pathways to evoke painful sensation, through which little other conscious sensation is encoded from the gut. Therefore it is necessary to understand the basis of nociception in these specific spinal pathways in order to study visceral pain in these conditions.

1.3 NEUROANATOMY OF THE GI TRACT

The transduction of physiological and noxious stimuli is mediated by the extrinsic innervation in the GI tract. This can be split into three main pathways, spinal thoracolumbar, spinal lumbosacral and vagal. Vagal afferents innervate a wide range of organs in the viscera, including the heart, trachea, liver, pancreas and upper and lower GI tract (Berthoud & Neuhuber, 2000). The density of vagal innervation decreases in a proximo-distal gradient throughout the GI tract, with minimal innervation in the distal colon (Berthoud, Jedrzejewska, &

Powley, 1990). In parallel, spinal thoracolumbar afferents innervate the oesophagus, stomach, small intestine and proximal colon via the great splanchnic and lesser splanchnic nerves. Spinal fibres of the lumbar splanchnic nerve innervate the mid to distal colon. The distal colon and rectum are principally innervated by the lumbosacral pelvic pathway although, as with all three pathways, anatomical overlap does occur (Figure 1).

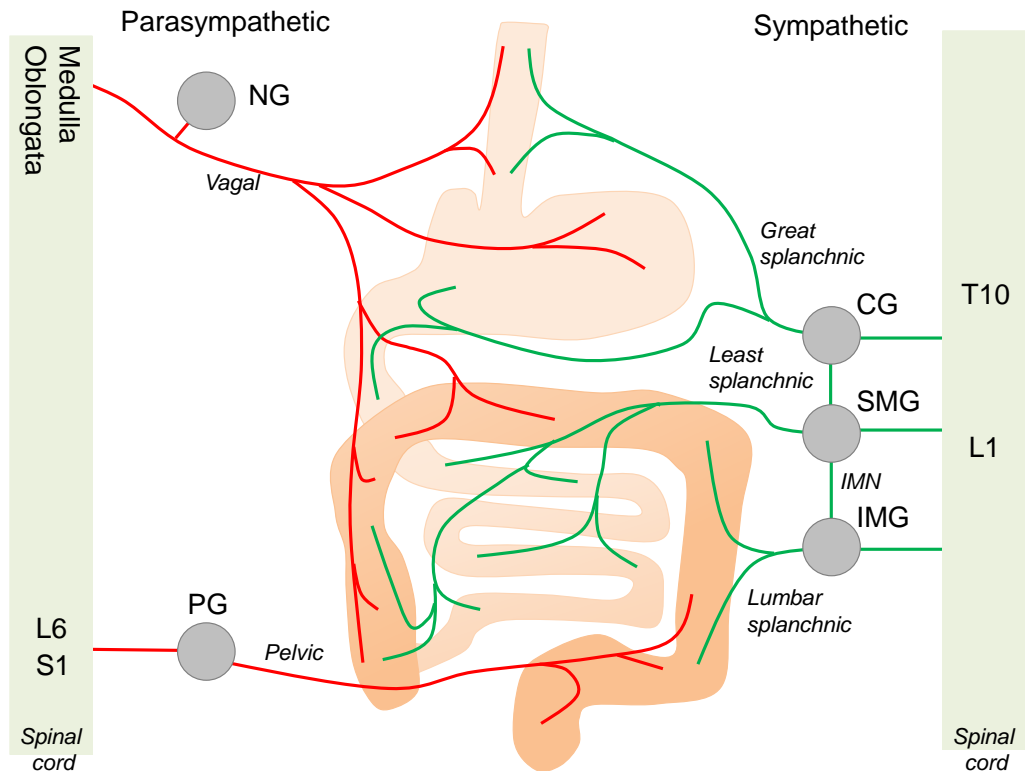


Figure 1. Neuroanatomy of the mammalian gastrointestinal tract. The three main afferent pathways innervating the gut are shown; vagal, thoracolumbar and lumbosacral. Vagal afferent fibres originate from cell bodies located in the nodose ganglia (NG) and project centrally to the medulla oblongata, and peripherally to the upper and lower GI tract. Thoracolumbar afferent fibres originate in dorsal root ganglia neurones (T7-L1) and project centrally to the dorsal horn and peripherally to the upper and lower GI tract. Thoracolumbar afferents pass through and arborise with, pre-vertebral sympathetic ganglia, including the celiac ganglia (CG), superior mesenteric ganglia (SMG) and inferior mesenteric ganglia (IMG). Lumbosacral afferents possess somata in dorsal root ganglia (L6 and S1), project centrally to the dorsal horn and project peripherally to the distal colon and rectum, passing through the pelvic ganglia (PG).

1.3.1 SPINAL AFFERENT INNERVATION OF THE GUT

The spinal innervation of the gut comprises of the thoracolumbar and the lumbosacral pathway. Their cell bodies reside in dorsal root ganglia (DRGs) in the thoracic, upper lumbar and sacral spinal vertebrae and project into the spinal cord and peripherally through sympathetic pre-vertebral ganglia to the gut wall (Cervero & Sharkey, 1988; Jänig & McLachlan, 1987). Centrally, afferents project to laminae I, II and V of the dorsal horn bypassing lamina III and IV (Figure 2)(Cervero & Connell, 1984). In some instances, they will project contralaterally to lamina V and X (De Groat, 1986). From here, second-order neurones in the spinal cord project to thalamic and parabrachial structures in the brain. The major spinal innervation of the gut is bimodally distributed across DRG vertebral levels, with peak innervation between thoracic 10 through to lumbar 1 (T10-L1; thoracolumbar) and lumbar 6 to sacral 1 (L6-S1; lumbosacral) (Christianson, Traub, & Davis, 2006; Robinson, McNaughton, Evans, & Hicks, 2004). Thoracolumbar afferent fibres innervate the mid-distal colon via the lumbar splanchnic nerve (Figure 1) and lumbosacral afferent fibres innervate the rectum, anus and very distal colon. Indeed, 3-15% of total DRG neurones project to the gut with the remainder terminating in superficial cutaneous or deep somatic tissues (Brierley et al., 2008; Cervero, Connell, & Lawson, 1984; Grundy, 2002; Jänig & Morrison, 1986; Perry & Lawson, 1998). Spinal afferents of the gut can be characterised by the location of their receptive field and their responses to mechanical stimuli (including von Frey hair probe, distension, circumferential stretch and light stroke). Specifically, distinct classes of afferent fibre can be identified based on these responses: muscular (those responding to low intensity circumferential stretch (≤ 5 g), but not fine mucosal

stroking); mucosal (those responding to light von Frey hair stroking of the mucosa), mesenteric (those responding to focal compression of the mesentery) and serosal (those responding to focal compression of the colon wall, but not mucosal stroking or low intensity circular stretch). Of these, serosal and mesenteric subtypes are believed to represent major populations of visceral nociceptors. In addition, a population of mechanically insensitive afferent fibres, which may become mechanical sensitive following inflammation, is also present in both thoracolumbar and lumbosacral pathways (Feng & Gebhart, 2011). The characterisation of all these afferent types, both functionally and morphologically, is discussed below. Their relative proportions also differ significantly between the thoracolumbar and lumbosacral pathways, and is likely to be representative of the different functional requirements of sensory innervation in these different gut regions (Brierley, Jones, Gebhart, & Blackshaw, 2004).

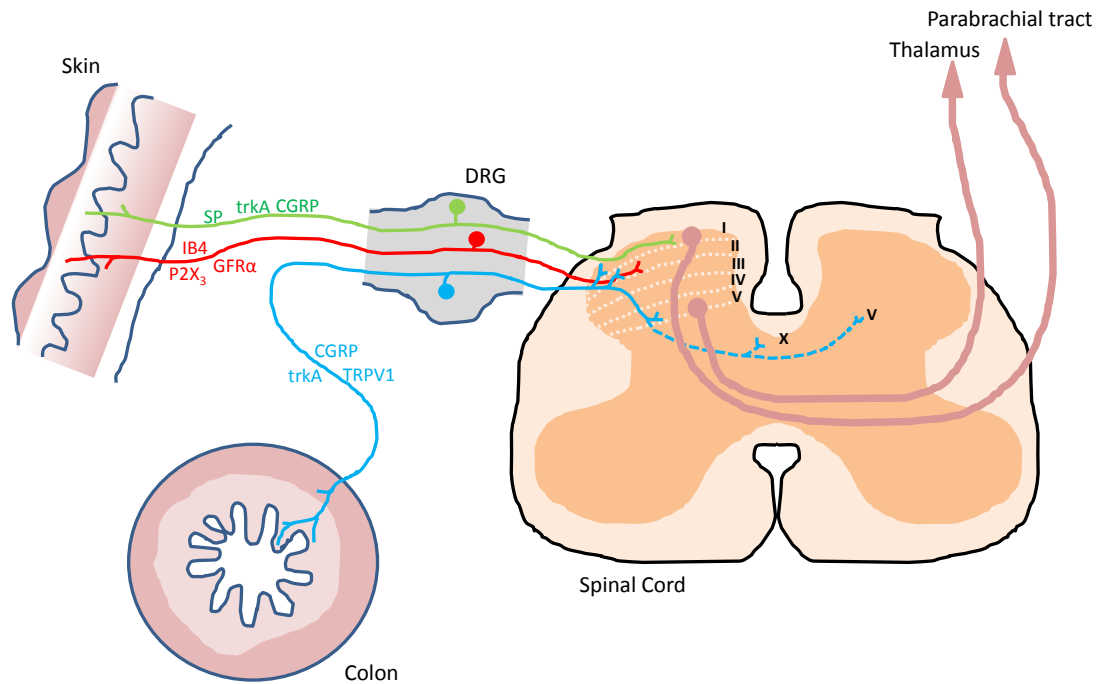


Figure 2. C-fibres (somatic and visceral) and the dorsal horn of the spinal cord. Schematic showing central terminations of the two main groups of somatic and visceral C-fibres in the spinal cord. Somatic C-fibres differ by their content of peptides, receptors and site of termination within the spinal cord. Peptidergic somatic C-fibres terminate in lamina I, whilst non-peptidergic C-fibres terminate in lamina II. Visceral C-fibres are predominantly peptidergic with diffuse and multiple central terminations in laminae I, II, V and X, some project contralaterally to laminae V and X (Hunt & Rossi, 1985; Sugiura, Terui, & Hosoya, 1989). (CGRP, calcitonin gene-regulated peptide; DRG, dorsal root ganglia; GFRα, glia cell line-derived neurotrophic factor family receptor alpha; IB4, IB4-lectin binding site; P2X₃, purinoceptor; SP, substance P; trkA, tyrosine receptor kinase A; TRPV1, transient receptor potential vanilloid 1).

1.3.2 SPINAL THORACOLUMBAR PATHWAY

1.3.2.1 Splanchnic serosal and mesenteric afferents

The encoding of traction and stretch of the mesentery (both established noxious stimuli) is achieved by subsets of afferent fibres which are strongly associated with mesenteric arteries branch points and blood vessels innervating the gut (Bessou & Perl, 1966; Jänig, 1996; Ray & Neill, 1947). These types of vascular afferents are also common to other viscera, including the spleen, ovary, bladder, and pancreas (Berthoud, 2004; Floyd & Morrison, 1974). As well as responding to compression of their receptive fields by von Frey hair probe, these afferents also encode much higher intensities of circumferential stretch and balloon distension (~50mmHg (Brierley et al., 2008; Hughes et al., 2009)) than so-called muscular afferents identified in the distal colon. They are also thought to comprise a major class of nociceptor, responsive to ischaemia, hypoxia and inflammatory insult (Haupt, Jänig, & Kohler, 1983; Longhurst & Dittman, 1987; Longhurst, Kaufman, Ordway, & Musch, 1984). These afferents have been sub-characterised into serosal and mesenteric based on the location of their receptive fields in flat-sheet *ex vivo* colonic preparations (Brierley et al., 2004). Anatomically in guinea-pig, afferent endings have not been localised to serosal layers, and it is likely that such serosal afferents correlate with sensory endings on intramural arterioles within the submucosa, however comparable studies have yet to be undertaken in other species (Song et al., 2009). As such the term 'serosal' afferent may represent a misnomer. It has been proposed that the term 'vascular' afferent be used to encapsulate both serosal and mesenteric afferent subtypes (Brookes, Spencer, Costa, & Zagorodnyuk, 2013; Zagorodnyuk,

Brookes, & Spencer, 2010). Whilst it is likely that 'serosal' may prove not to be the best descriptor of this afferent subtype, there remain significant functional differences between those afferents terminating in the mesentery and in the intramural colon wall. For example, they are differently altered in ASIC2 ^{-/-} mice, as well as having different wild-type sensitivities to distension and von Frey hair probe (Page, Brierley, et al., 2005; Song et al., 2009). This suggests that specialisation may exist within vascular afferents, dependent on the localisation of the receptive field, or that the local environment infers specific functional characteristics. What is clear is that serosal and mesenteric afferents populate the majority of the splanchnic innervation of the colon (80-90% (Brierley et al., 2004)) and that these afferents are functionally distinct to both muscular and mucosal afferent subtypes.

Importantly, as well as providing mechano-transduction sites, serosal and mesenteric afferents express receptors for a vast array of chemical mediators, including ATP, bradykinin, glutamate, nerve growth factor, histamine, prostaglandins and 5-HT (Blackshaw & Gebhart, 2002; Brierley, Carter, et al., 2005; Brunsden & Grundy, 1999; Grundy, 2004; Hicks et al., 2002; Maubach & Grundy, 1999; Rong, Spyer, & Burnstock, 2002). They are also sensitive to inflammatory cytokines including IL-1 β , IL-6 and TNF- α (Andratsch et al., 2009; Binshtok et al., 2008; Y. Li, Ji, Weihe, & Schäfer, 2004). Indeed, mechanically insensitive afferents, which make up almost a third of thoracolumbar spinal afferents innervating the colon and are thought to represent a population of 'silent' serosal-like afferents, may be sensitised by addition of inflammatory

mediators and become mechanically sensitive (Feng & Gebhart, 2011; Feng, La, Schwartz, et al., 2012).

1.3.2.2 Splanchnic muscular afferents

Limited anatomical evidence exists detailing the peripheral projections of dorsal root ganglia afferents; however this has been examined for the gastro-oesophageal junction and pylorus (Clerc & Mazzia, 1994; Lindh, Aldskogius, & Hökfelt, 1989). Functionally, spinal afferent innervation of the oesophagus is split into wide dynamic range mechanoreceptors that encode into noxious distension ranges (threshold $\sim 3\text{mmHg}$), and high threshold ($>30\text{mmHg}$) mechanoreceptors (Sengupta & Gebhart, 1994; Sengupta, Saha, & Goyal, 1990). In the colon, muscular afferents, which respond to low intensity stretch, make up $\sim 10\%$ of mechanosensory afferents (Brierley et al., 2004). It is possible that muscular afferents represent a population of distally-projecting wide dynamic range mechanoreceptors comparable to those observed in the oesophagus. Currently, no morphological characterisation of these fibre types has been undertaken and questions still remain over their likely function. That they encode across a range of stimulus intensities into the noxious range suggests they will play a role in nociception. However, their contribution and therefore impact on whole-nerve afferent outflow from the distal colon will be limited by the relative low population of this afferent subtype.

1.3.2.3 Splanchnic mucosal afferents

Mucosal afferents have been reported in recordings from the splanchnic nerves of both the mouse (4% (Brierley et al., 2004)) and rat colon (23% (Lynn & Blackshaw, 1999)). These are not responsive to stretch but are to light stroking

with von Frey hairs. These afferents are sensitive to 5-HT (Hicks et al., 2002), however their characterisation in the presence of other chemical mediators and during inflammation has not been extensively studied (Brierley et al., 2004; Hughes et al., 2009). It is unlikely that splanchnic mucosal afferents are involved in nociceptive signalling pathways.

1.3.3 LUMBOSACRAL AFFERENTS

The lumbosacral pelvic afferents compose the second major spinal innervation of the colon; characterisation of these afferents has revealed a number of functionally distinct subtypes comparable to those seen in the thoracolumbar pathway. These spinal afferent endings were originally described as having unspecialised bare nerve endings (Cervero, 1994; Jänig, 1996) but have recently been suggested to possess morphological specialisation in some pelvic afferent endings and may resemble specialised vagal endings (Lynn & Brookes, 2011; Lynn, Olsson, Zagorodnyuk, Costa, & Brookes, 2003; Olsson, Costa, & Brookes, 2004). Specifically, lumbosacral pelvic afferents regulate complex defecatory processes as well as being the primary pathway mediating experimental visceromotor reflexes following colorectal distension paradigms (Kyloh, Nicholas, Zagorodnyuk, Brookes, & Spencer, 2011).

1.3.3.1 Rectal intraganglionic laminar endings (rIGLEs)

Structures termed rectal intraganglionic laminar endings, which are comparable to vagal intraganglionic laminar endings (IGLEs) with basket-like endings encapsulating myenteric ganglia, have been identified in lumbosacral pelvic nerves that terminate at rectal myenteric ganglia in guinea pig (Olsson et

al., 2004). Spinal rectal IGLs seem capable of encoding stretch over a wide dynamic range with a low-threshold of activation (Lynn et al., 2003). In this respect, they are comparable to muscular subtypes and are likely representative of pelvic muscular afferents recorded from pelvic nerve of cat, rat and mouse, which are responsive to relatively low levels of distension/stretch (Brierley et al., 2004; Jänig & Koltzenburg, 1991; Sengupta & Gebhart, 1994). These afferents are probably responsible for the transduction of important parasympathetic defecatory reflexes, but also contribute to encoding noxious stimuli. Following anterograde dye fills of pelvic nerves, rIGLE-like structures were not clearly distinguishable in mouse myenteric ganglia of the colorectum (Spencer, Kerrin, Singer, et al., 2008), suggesting that, at least, morphologically these structures may be species-dependent.

1.3.3.2 Pelvic and rectal intramuscular endings

Anterograde labelling of nerves innervating the colorectum have identified significant afferent arborisation within both circular and longitudinal muscle layers (Olsson et al., 2004). In mice that lack enteric ganglia, there remains a subset of mechanoreceptors that transduce stretch stimuli, which suggests that a population of intramuscular rectal mechanoreceptors exists, independent of endings arborising at rectal myenteric ganglia (rIGLEs) (Spencer, Kerrin, Zagorodnyuk, et al., 2008). Functionally, in mouse, muscular wide dynamic range afferents that encode innocuous and noxious stimuli intensities have been reported to make up approximately 21% of pelvic innervation of the colon (Brierley et al., 2004). Whether this corresponds to a combination of rIGLE- and rectal intramuscular ending-like afferents is currently unknown. Anatomical

structures of rectal intramuscular ending-like afferents have been identified in the guinea pig external anal sphincter, which is in symmetry to the localisation of vagal intramuscular endings within sphincters of the upper GI (Berthoud, Patterson, Neumann, & Neuhuber, 1997; Lynn & Brookes, 2011). Very little physiological data exists on the putative function of these receptors in the distal colon and rectum, however their ability to encode across the full spectrum of stimuli intensities suggests their sensitisation may be relevant to inflammatory pain.

1.3.3.3 Mucosal and muscular-mucosal afferents

Of the total mechanoreceptive fibres in the mouse pelvic nerve, approximately 20% are characterised as mucosal afferents, which are sensitive to light mucosal stroking but not circumferential stretch (Brierley et al., 2004). A further subtype of muscular-mucosal afferents represent a group of fibres that are activated by both light mucosal distortion, and by stretch or contraction of the bowel and make up about a quarter of pelvic fibres (Brierley et al., 2004). No such muscular-mucosal afferent subtype exists in the splanchnic thoracolumbar pathways (Brierley et al., 2004). The transduction of stretch and light stroke stimuli in muscular-mucosal afferents may occur at distinct separate sites, or alternatively, endings that are located in the sub-epithelial plexus may be capable of transducing both stimuli (Brierley et al., 2004; Brookes et al., 2013). Whilst this remains to be determined, muscular-mucosal afferents likely detect movement of luminal content over the surface of the GI tract, and as such may be relevant to the development of visceral hypersensitivity.

1.3.3.4 Pelvic serosal afferents

Finally serosal-like afferents can be observed in recordings from the pelvic nerve and contribute to approximately a quarter of total fibres (Brierley et al., 2004). These fibres are responsive to focal compression with von Frey hair probe and also to very high levels of circumferential stretch and distension pressures (Brierley et al., 2004; Brierley et al., 2008; Hughes et al., 2009). In addition, almost a quarter of pelvic lumbosacral afferents are mechanical insensitive and are likely to represent a population of 'silent' pelvic serosal afferents. Like thoracolumbar counter-parts, these may be mechanically sensitised by addition of an inflammatory soup (Feng & Gebhart, 2011). No lumbosacral equivalent of mesenteric afferents exists in the pelvic nerve (Brierley et al., 2004), suggesting that noxious mesenteric traction or stretch is not perceived by the distal colorectum.

1.3.4 VAGAL PATHWAYS

In conjunction with the spinal extrinsic innervation of the colon, vagal afferents also innervate visceral organs, conveying predominantly physiological stimuli such as satiety and nausea, but may also encode noxious stimuli (Grundy, 2002; S. Yu, Undem, & Kollarik, 2005). Vagal cell bodies are located in the nodose ganglia and project centrally to supraspinal pathways in the brain stem, hypothalamus and amygdala; structures associated with the behavioural and emotional aspects of sensory processing (Jänig, 1996). Whilst vagal afferents primarily transduce innocuous stimuli, they are believed to play a modulatory role in the central inhibitory control of nociception and pain. For example,

responses to noxious thermal sensation in rat hind-paw can be inhibited or enhanced through vagal afferent stimulation (Khasar, Green, Miao, & Levine, 2003; Randich & Gebhart, 1992). There are three morphologically distinct endings found on vagal afferents: mucosal, intraganglionic laminar endings (IGLEs) and intramuscular endings (Berthoud et al., 2001; Berthoud & Neuhuber, 2000; Berthoud et al., 1997; Berthoud & Powley, 1992; Neuhuber, 1987; F. B. Wang & Powley, 2000). Functional electrophysiological recordings have identified three types of vagal afferent in the upper GI tract that are responsive to differing stimuli; these are mucosal (responsive to mucosal stroking and luminal chemical stimuli), low threshold stretch-sensitive muscular (stimulated during contraction and distension of the bowel and saturating within physiological levels of distension) and high threshold distension-sensitive afferents (IGGO, 1955; Ozaki, Sengupta, & Gebhart, 1999; Page, Martin, & Blackshaw, 2002; Sengupta et al., 1990; S. Yu et al., 2005; Zagorodnyuk, Chen, Costa, & Brookes, 2003). The evidence for building structure-function relationships based on these data is outlined below.

1.3.4.1 Vagal intraganglionic laminar endings

As described for lumbosacral pathways, vagal intraganglionic laminar mechanoreceptors (IGLEs) are morphologically comparable to anatomical structures in the rectum. Specifically, IGLEs possess basket-like endings that encapsulate myenteric ganglia, interacting with neuronal and non-neuronal tissues (E. A. Fox, Phillips, Martinson, Baronowsky, & Powley, 2000). They act as tension-sensitive mechanoreceptors and are found at all vagal afferent-myenteric plexi interfaces, from the oesophagus through to the distal colon,

although less extensively in more distal regions (Berthoud et al., 1997; E. A. Fox et al., 2000; F. B. Wang & Powley, 2000). They likely transduce the compression of the ganglia by surrounding gut wall layers and signal gastric distension after a meal (Berthoud & Neuhuber, 2000). These mechanoreceptors are significantly modulated by biological mediators, including the neurotransmitters, ATP, glutamate and GABA, and the hormone ghrelin, which are capable of both excitation and inhibition of IGLEs (Kentish et al., 2012; Page, Young, et al., 2005; Smid, Young, Cooper, & Blackshaw, 2001; Zagorodnyuk et al., 2003). Vagal IGLEs are low-threshold, slow-adapting afferents that distally respond to physiological distension of the gut caused by the normal movement of gastrointestinal contents. Responses by vagal IGLEs in electrophysiological recordings of distended rat jejunum initiate and plateau within the first 10-20mmHg (Booth, Kirkup, Hicks, Humphrey, & Grundy, 2001). These properties, of intense responses saturating at innocuous low distension thresholds, are consistent with previously described vagal 'tension mechanoreceptors', which at least in the guinea pig oesophagus are representative of nodose A-fibres (Blackshaw, Page, & Partosoedarso, 2000; Sengupta, Kauvar, & Goyal, 1989; S. Yu et al., 2005). These characteristics differ from rIGLEs, which seem capable of encoding over a wide dynamic range and may reflect pathway-specific differences. As such, vagal IGLEs are unlikely to encode noxious stimuli from the GI tract.

1.3.4.2 Vagal intramuscular endings

In circular and longitudinal muscle, vagal afferents branch and run in parallel to smooth muscle nerve bundles forming intramuscular arrays (IMAs) (Berthoud

& Powley, 1992; E. A. Fox et al., 2000). IMAs have a distinct distribution within the GI tract with predominance in the fundus, lower oesophageal sphincter and pyloric sphincter (F. B. Wang & Powley, 2000). IMAs have been purported to detect stretch rather than tension during distension events but there remains limited functional evidence to support this (Phillips & Powley, 2000). A population of mechanosensitive vagal afferents with wide dynamic range and graded firing into noxious distension pressures in the oesophagus may in fact possess IMA-like endings and represent a population of nociceptive fibres (S. Yu et al., 2005). However, disparity between the anatomically distinct distribution of IMAs (e.g. fundus, lower oesophageal sphincter and pyloric sphincter) and the relatively extensive presence of wide dynamic range mechanosensitive afferents along the guinea pig oesophagus, suggests that functions attributed to IMAs should be applied cautiously.

1.3.4.3 Vagal mucosal afferents

Anterograde tracings from nodose ganglia have identified non-specialised endings in villi of the mid-gut (Berthoud, Kressel, Raybould, & Neuhuber, 1995; R. M. Williams, Berthoud, & Stead, 1997). These vagal mucosal afferents are sensitive to gentle stroking or compression of the mucosa, but not distension or stretch (Page & Blackshaw, 1998). Vagal mucosal afferents are often strongly associated with enteroendocrine cells found in the mucosa and are activated by mediators released from these cells, including 5-hydroxytryptamine and bile salts (Grundy, 2004; Page et al., 2002). The modulation of vagal mucosal afferent terminals acts to regulate satiety, occurring predominantly through the release of enteroendocrine mediators including cholecystokinin (CCK),

glucagon-like peptide 1 (GLP1), apolipoprotein A-IV, enterostatin, gastrin-releasing peptide, oxyntomodulin, amylin and peptide YY (Grundy, 2004). Mucosal afferent sensitivity to compression of the mucosa enables the transduction of food bolus movements along the upper gut but is unlikely to contribute to nociceptive signalling.

1.3.5 CONSOLIDATION OF FIBRE SUBTYPES

As outlined above, earlier investigators have attempted to define visceral afferents into discrete subtypes, based on structure and function (Brierley, 2010; Grundy, 2002; Leek, 1977). The heterogeneity of visceral afferents under investigation throughout the GI tract, and differences in *in vivo* and *in vitro* mechanical stimuli used to characterise afferents, has resulted in non-standardised nomenclature. A recent review by Brookes *et al.* has attempted to consolidate earlier classifications into five major afferent subtypes that encompass all the afferent fibres of the vagal, thoracolumbar and lumbosacral anatomical pathways (Brookes et al., 2013). The five afferent subtype model includes (1) intraganglionic laminar mechanoreceptors, (2) mucosal afferents, (3) muscular-mucosal afferents, (4) intramuscular afferents and (5) vascular afferents (Figure 3).

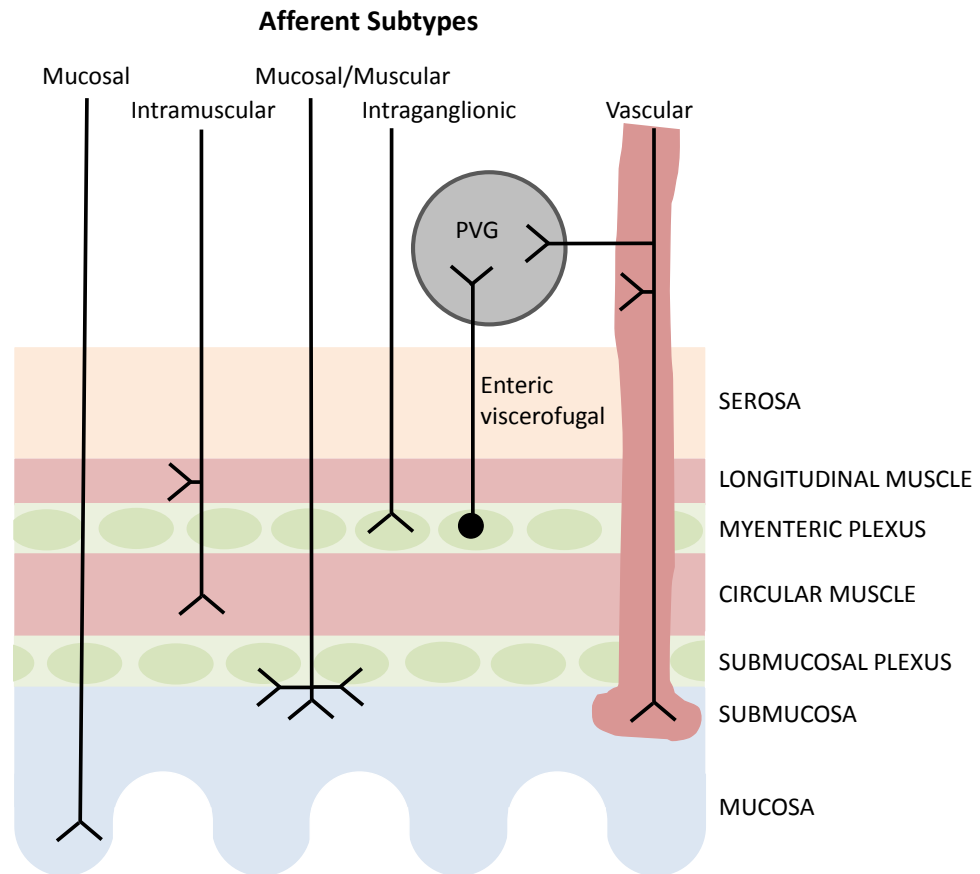


Figure 3. Visceral afferent innervation of the gastrointestinal tract.
Five afferent subtype model. Vagal and spinal extrinsic pathways and intrinsic viscerofugal innervation of prevertebral ganglia (PVG).

Whilst this approach is undoubtedly the most appropriate, limited evidence exists where function has been successfully attributed to structural characteristics (Lynn et al., 2003; Song et al., 2009; Spencer, Kerrin, Singer, et al., 2008). Further still, challenges clearly exist in the translation of structural findings between species, especially into murine models, where anatomical structures appear less well defined. As such, it is likely that this model represents an oversimplification of what is a complex system. Importantly, and in the context of IBD, the thoracolumbar anatomical pathway, and specifically, serosal and mesenteric afferent subtypes can be considered the major nociceptive signalling pathway in these conditions.

1.3.6 EFFERENT FIBRES

The vagal and spinal nerves described above also contain an efferent component. These are relevant as they coordinate the regulation of visceral organs and have a direct impact on afferent activity and *vice versa*. In the vagus, efferent fibres, which are outnumbered 9 to 1 by afferent fibres, have their cell bodies in the dorsal motor nucleus and nucleus ambiguus of the brain stem (Jänig, 1996; Leek, 1977). In the splanchnic and pelvic nerves, afferent:efferent ratios are 3:1 and 1:1, respectively (Leek, 1977). The efferent sympathetic innervation of the gut is formed from the 'sympathetic chain' of pre-vertebral ganglia (PVG), of which three are important to the lower GI tract: the inferior mesenteric (IMG), the superior mesenteric (SMG) and the celiac (CG) ganglia. Efferent neurones are responsible for the regulation of motility, secretion, absorption and blood flow to these tissues. In efferent function, the two spinal nerves differ greatly, with the pelvic nerve forming the major parasympathetic

innervation and the splanchnic nerve being the prominent sympathetic innervation to the lower GI tract. Collaterals of afferent fibres passing through pre-vertebral ganglia arborise with cell bodies residing there and provide local feedback loops to regulate bowel function.

1.3.7 INTRINSIC NEURONAL PROJECTIONS

In addition to extrinsic innervation, within the myenteric and submucosal layers of the gut wall resides a dense network of neurones that form the intrinsic enteric nervous system (ENS). These neurones are responsible for the local control and complex coordination of GI motor and secretory function (Costa, Brookes, & Hennig, 2000). Specifically, they regulate local blood flow, mucosal transport and secretion, motor, immune and endocrine function (Costa et al., 2000). The ENS is critical for myogenic rhythmic activity, migrating motor complexes and content-dependent functions, such as the accommodation and propulsion of luminal contents. A reflexive feedback loop exists from the gut to the sympathetic chain, with retrograde tracer studies labelling a population of neurones that reside in the intestine and project to pre-vertebral ganglia (Miller & Szurszewski, 1997) (Figure 3). Such viscerofugal fibres provide important feedback from enteric neurones on gut volume to sympathetic pathways, and mark the pre-vertebral ganglia as sites of coordination mediating mechano-gastrointestinal function (Szurszewski, Ermilov, & Miller, 2002). These fibres are important to note as electrophysiological recordings from the lumbar splanchnic nerve may contain small proportions of these fibres (Luckensmeyer & Keast, 1995). Another class of viscerofugal fibres that may influence lower GI function are rectospinal neurones. These fibres, that originate in the myenteric

plexus and project to the spinal cord, have been reported to participate in the defecation reflex, although their physiological stimulus is not fully understood (Neuhuber et al., 1993).

1.4 CHARACTERISATION OF VISCERAL NOCICEPTORS

In the viscera, as elsewhere, the encoding of noxious, high intensity and normally painful stimuli is undertaken by nociceptors, which represents a heterogeneous population of afferent fibres. In somatic pathways, certain characteristics and markers identify nociceptors from non-nociceptive neurones. These include electrophysiological characteristics and a correlate between cell size, myelination and conduction velocity (CV), and the expression of protein markers. In the viscera, such correlations are poorly defined and classical nociceptive markers or characteristics must be applied with care. Indeed, significant populations of visceral afferents are intensity encoding and transduce both noxious and physiological stimuli, and therefore are not specialised to a solely nociceptive phenotype.

Somatic nociceptive neurones develop significantly broader action potentials (AP), have larger AP overshoots and longer afterhyperpolarisation than non-nociceptive neurones (Fang, McMullan, Lawson, & Djouhri, 2005). Furthermore, nociceptive neurones fall into two predominant categories of fibre type; unmyelinated C-type, with CVs <2.0 m/s, and thinly myelinated A δ -type, with CVs of 2-30 m/s (Fang, McMullan, et al., 2005). Faster conducting (>30 m/s), and therefore more highly myelinated, A α / β fibres, almost exclusively encode non-noxious stimuli (Fang, McMullan, et al., 2005). Axon myelination state and conduction velocity is significantly correlated to soma size and axonal diameter,

with small somal neurones having unmyelinated slow conducting axons (Harper & Lawson, 1985a; Yoshida & Matsuda, 1979). Whilst conduction velocity is not a perfect predictor of nociceptive phenotype, less than 10-20% of C-fibre and A δ -fibre neurones have been characterised as non-nociceptive (Fang, McMullan, et al., 2005).

Protein markers have also been shown to associate with nociceptive neuronal phenotypes and may be used to identify putative nociceptors. Such markers include trkA (a receptor for nerve growth factor(NGF)), IB4 binding (the isolectin B4 isolated from *Griffonia simplicifolia* that binds specific sugar residues), CGRP (calcitonin gene-related peptide), TRPV1 (transient receptor potential V1 receptor) and P2X₃ receptor expression (Fang, Djouhri, et al., 2005; Fang et al., 2006; Hunt & Mantyh, 2001; Wirkner, Sperlagh, & Illes, 2007).

Classically, unmyelinated C fibres can be split into two broad classes; a population that expresses trkA, the receptor for NGF, and contains peptidergic neurotransmitters, such as substance P and CGRP; and the non-peptidergic population, which do not express peptidergic neurotransmitters or trkA, but do have P2X₃ positive immunoreactivity and bind isolectin B4 (Hunt & Rossi, 1985).

In the viscera, the same distinctions do not exist. Visceral projections are almost exclusively unmyelinated C-fibres with some thinly myelinated A δ fibres (20-40%) and vagal afferents are considered to be exclusively unmyelinated C-fibres (<2m/s)(Cervero & Sharkey, 1988; Malin, Christianson, Bielefeldt, & Davis, 2009; Ozaki et al., 1999; Sengupta & Gebhart, 1994; Sengupta et al., 1990). As such, the lack of a discrete innocuous pathway in the viscera

necessitates the encoding of physiological stimuli by C- and A δ -fibres. Furthermore, viscerally projecting neurones are almost exclusively peptidergic and have high expression of CGRP and TRPV1 compared to somatic neurones (Christianson et al., 2006; Robinson et al., 2004; Wall & Melzack, 1999). The purportedly nociceptive marker of IB4 binding is significantly less in the viscera at 5-20% of colonic DRG neurones, and those that are IB4-positive almost exclusively co-label with CGRP (Christianson et al., 2006; Robinson et al., 2004). This contrasts to somatic populations where CGRP and IB4 are predominantly thought to represent mutually exclusive C-fibre populations. In the absence of extensive evidence linking functionally defined visceral nociceptors to specific protein markers, the translation of protein markers associated to somatic nociceptors onto visceral populations should be treated with caution. As such, visceral afferents of a nociceptive phenotype are typically defined by their functional responses or receptive field location (e.g. the mesenteric subtype).

1.5 REGULATING VISCERAL AFFERENT EXCITABILITY

The encoding of noxious stimuli occurs through the propagation of action potentials from the peripheral nerve terminal of a visceral afferent to the cell body and central arbor. Importantly, in some patient groups, the therapeutic inhibition of mechanosensitivity in visceral afferents has proven to be efficacious in treating pain (Verne, Robinson, Vase, & Price, 2003; Verne, Sen, & Price, 2005). Specifically, topical rectal application of lidocaine is capable of treating visceral pain associated with IBS. This suggests that pharmacological modulation of excitability in visceral afferents is an approach likely to be successful in the development of novel analgesics. Neurones are maintained at a

constant polarised state of approximately -60mV (resting membrane potential; RMP) by the active movement of ions (including Na⁺ and K⁺) across the plasma membrane to generate a potential gradient. A variety of receptors and channels encode external cues into changes in membrane potential that may ultimately lead to the trigger of an action potential (AP) and depolarisation of the cell.

Visceral neurones are heterogeneous with respect to their sensitivity to differing stimuli. The relative likelihood of initiating an AP in any given cell is dependent on its state of excitability. This sensitivity to stimuli is plastic and prone to modulation, especially during inflammation (Brierley, Carter, et al., 2005; Brierley, Jones, Xu, Gebhart, & Blackshaw, 2005; Feng & Gebhart, 2011; Feng, La, Schwartz, et al., 2012; Hughes et al., 2009). Indeed, a subset of previously mechanically insensitive afferents, which make up approximately a third of thoracolumbar spinal afferents (Feng & Gebhart, 2011), may be sensitised by addition of an inflammatory soup (consisting of bradykinin, 5-HT, histamine, prostaglandin E2 and protons (pH 6.0)) to the receptive field.

Experimental colitis is also associated with sensitisation of colonic afferents and to *in vivo* visceral hypersensitivity to distension (Feng, La, Schwartz, et al., 2012; Feng, La, Tanaka, et al., 2012; Hughes et al., 2009), with such changes in sensitivity the product of the modulation of molecular receptors and channels present on visceral afferents.

Several candidate mechanisms have been identified that are known to regulate sensory neuronal excitability (including T-type calcium channels, TRP channels and HCN2)(Caterina et al., 1997; Emery, Young, Berrocoso, Chen, & McNaughton, 2011; Sekiguchi & Kawabata, 2013). In addition, voltage-gated

sodium and potassium channels are critical to the generation and propagation of the AP, and significantly contribute to visceral afferent excitability.

1.5.1 ROLE OF VOLTAGE GATED SODIUM CHANNELS IN ACTION POTENTIAL GENERATION

The activity of voltage gated sodium channels (Nav) underpins electrogenesis in excitable cells. The nine genes (*SCN1A-SCN5A* and *SCN8A-SCN11A*) encoding the Nav1.1-Nav1.9 α -subunit isoforms exhibit regional and temporal distributions and describe membrane proteins of varying biophysical characteristics (Goldin et al., 2000). A tenth α -subunit (Nav encoded by *SCN7A*) lacks the voltage sensor and is involved in sodium homeostasis (Goldin et al., 2000). The Na⁺ currents generated by these channels contribute significantly to the resting membrane potential (RMP) and principally to the rising phase of the action potential. Each voltage-gated sodium channel subtype contributes to the regulation of neuronal excitability in different ways. Pore-forming α -subunits are structured as four homologous domains (I-IV) each consisting of six α -helical transmembrane segments (S1-S6), with a re-entrant loop partially entering transmembrane regions between segments S5 and S6 (Catterall, 2000). Extracellular loops connect the re-entrant loop with either S5 or S6 of each domain and can interact with other transmembrane proteins. Significantly larger intracellular loops describe an inactivation region, phosphorylation modulation sites and connect the four domains. Whilst expression of the isolated α -subunit is capable of producing the necessary characteristics of a sodium channel (i.e. voltage-dependent activation, rapid inactivation and selective ion conductance (Hodgkin & Huxley, 1952)), most α -subunits require co-expression with auxiliary membrane proteins to develop physiologically-relevant currents (P. B.

Bennett, Makita, & George, 1993; Isom, De Jongh, & Catterall, 1994; Isom et al., 1992; Isom et al., 1995). Importantly, the formation of sodium channel complexes with auxiliary membrane proteins, such as β -subunits, is cell-type specific. For example, skeletal muscle α -subunits (Nav1.4) have altered gating in the absence of β -subunits (P. B. Bennett et al., 1993), by contrast, the influence of β -subunits on channel function of Nav1.6 α -subunits in sensory neurones is relatively weak (Zhao, O'Leary, & Chahine, 2011). A number of other proteins interact with sodium channels, including p11, ankyrin and Nedd4, to influence current characteristics and channel expression (Okuse et al., 2002; Shao, Okuse, & Djamgoz, 2009; Zhao et al., 2011). Together these data highlight the importance of the cellular environment in the functional expression of sodium channel currents.

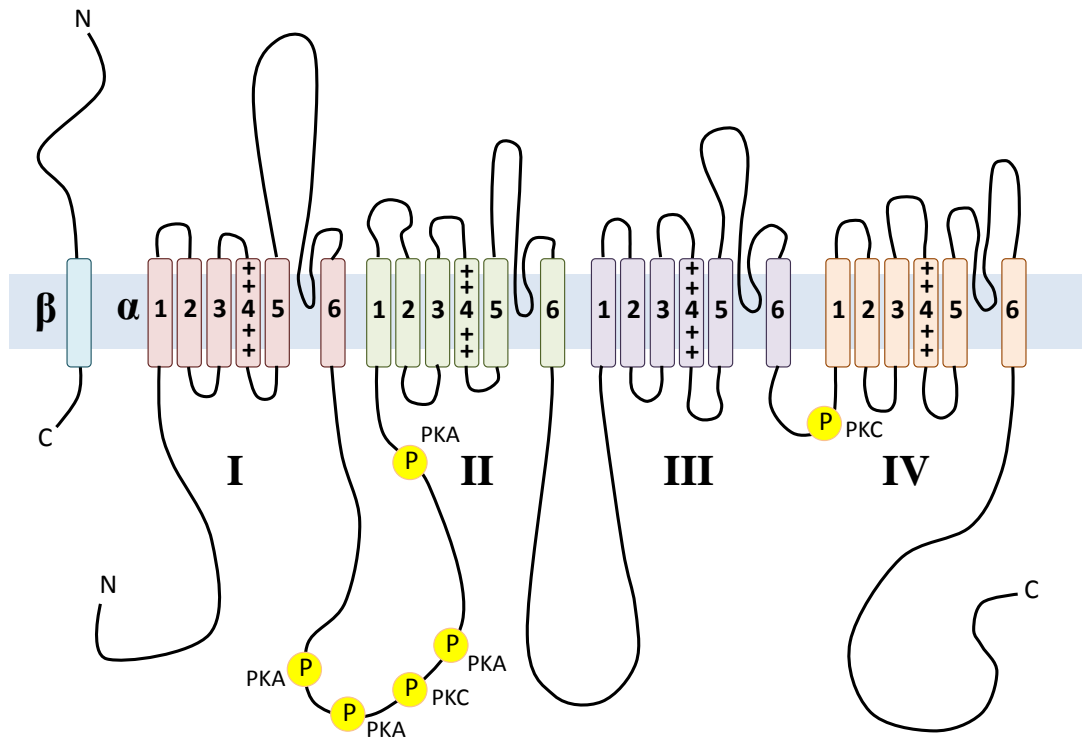


Figure 4. Voltage-gated sodium channel (Na_v) schematic.

The four domain α-subunit has conserved intracellular phosphorylation sites which are substrates of Protein Kinase A and C (PKA & PKC). The α-subunit can couple to one of four β-subunits localising the channel in the membrane.

In broad terms, sodium channel α -subunits can be characterised by their tissue of primary expression. For Nav1.1, Nav1.2, Nav1.3 and Nav1.6 this is considered to be neurones of both the central nervous system (CNS) and peripheral nervous system (PNS). Nav1.4 and Nav1.5 are expressed by skeletal muscle and cardiac muscle myocytes, respectively. Finally, Nav1.7, Nav1.8 and Nav1.9 are expressed primarily in neurones of the PNS. Nonetheless, most channels will have some expression in other tissues. As such, multiple sodium channels coordinate to regulate the relative excitability of any individual cell. The comparative importance of specific α -subunits to excitability in different cell types is clear from the clinical manifestations of known sodium channelopathies (see Table 2). For example, mutations in skeletal Nav1.4 cause hyperkalemic periodic paralysis and paramyotonia congenita in familial periodic paralysis syndromes (McClatchey et al., 1992; Ptáček et al., 1992; Ptáček et al., 1991; Rojas et al., 1991). Long QT syndrome type 3 is produced by cardiac Nav1.5 mutations resulting in impaired inactivation and hyperexcitability (P. B. Bennett, Yazawa, Makita, & George, 1995; Q. Wang et al., 1995). Pain phenotypes (such as primary erythromelalgia, paroxysmal episodic pain syndrome, painful neuropathy and congenital insensitivity to pain) have been associated with mutations arising in peripherally expressed sodium channels (Nav1.7 (Fischer & Waxman, 2010), Nav1.8 (Faber et al., 2012; Huang et al., 2013) and Nav1.9 (Huang et al., 2014; Leipold et al., 2013; Zhang et al., 2013). Furthermore, mutations in the auxiliary β 1 subunit is causal in inherited febrile seizures directly modulating brain sodium channels (Wallace et al., 1998). The importance of this cell type specific expression was elegantly demonstrated by Rush *et al.*, where a single mutation in Nav1.7 (F1449V) which manifests

clinically as primary erythromelalgia, can cause *hyperexcitability* in dorsal root ganglia (DRG) neurones and *hypoexcitability* in sympathetic neurones (Rush et al., 2006). These differential functional effects of the mutation go some way to explain the varied symptoms present in these patients. The co-expression of Nav1.8 in DRG, but not sympathetic, neurones is a major contributor to this effect, due to its availability for activation at depolarised membrane potentials (Rush et al., 2006). Therefore, the actions of a single α -subunit should be taken in context, not only with its colocalisation with auxiliary subunits but also with the expression of other α -subunits within the cell. This has important implications when inferring the functional consequences of sodium channel expression across diverse cell types.

Type	Gene	Primary tissue	Major inherited diseases	References
Nav1.1	<i>SCN1A</i>	CNS	Generalised epilepsy with febrile seizures Dravet syndrome (severe myoclonic epilepsy in infancy) Benign neonatal convulsions Familial hemiplegic migraine type III Benign familial neonatal-infantile seizures	Reviewed in (Catterall, Kalume, & Oakley, 2010)
Nav1.2	<i>SCN2A</i>	CNS		(Heron et al., 2002)
Nav1.3	<i>SCN3A</i>	CNS		
Nav1.4	<i>SCN4A</i>	Skeletal muscle	Hypokalaemic periodic paralysis type II K ⁺ -sensitive normokalaemic periodic paralysis Hyperkalaemic periodic paralysis Paramyotonia congenita	(Struyk & Cannon, 2007) (Sokolov, Scheuer, & Catterall, 2008) (Ptáček et al., 1991; Rojas et al., 1991) (Ptáček et al., 1992)
Nav1.5	<i>SCN5A</i>	Cardiac muscle	Long QT syndrome type III Brugada syndrome	(P. B. Bennett et al., 1995; Q. Wang et al., 1995) (Terrenoire, Simhaee, & Kass, 2007)
Nav1.6	<i>SCN8A</i>	CNS		
Nav1.7	<i>SCN9A</i>	PNS	Erythromelalgia Paroxysmal extreme pain disorder Congenital insensitivity to pain Anosmia Bladder pain syndrome	Reviewed in (Fischer & Waxman, 2010) (Fertleman et al., 2006) (Cox et al., 2006; Goldberg et al., 2007) (Weiss et al., 2011) (Reeder et al., 2013)
Nav1.8	<i>SCN10A</i>	DRG neurones	Painful neuropathy	(Faber et al., 2012; Huang et al., 2013)
Nav1.9	<i>SCN11A</i>	PNS	Congenital insensitivity to pain Familial episodic pain Painful neuropathy	(Leipold et al., 2013) (Zhang et al., 2013) (Huang et al., 2014)
NavX	<i>SCN6A</i>	Astrocytes, hypothalamus		

Table 2. Mammalian sodium channel α subunits and major inherited diseases.

1.5.2 SODIUM CHANNEL EXPRESSION PLASTICITY

As well as regional specificity in expression, sodium channels are also under significant temporal regulation. During development and following pathogenesis, sodium channel isoform expression displays significant plasticity. This is particularly relevant in the visceral hypersensitivity to colorectal distension observed in some patients, where functional up-regulation of sodium channel currents following inflammation contribute to visceral afferent hyperexcitability to non-noxious stimuli. In parity to other proteins, these alterations in expression are regulated at the transcriptional, translational and post-translational level. Analysis of 5' flank regions of Navs have revealed multiple sites for transcriptional regulation (Schade & Brown, 2000; P. Yang, Kupersmidt, & Roden, 2004), with examples of conservation between α -subunits (Martin, Tang, Ta, & Escayg, 2007). Importantly, growth factors and hormones can developmentally modulate Nav transcription (Boixel, Gavillet, Rougier, & Abriel, 2006; Fjell et al., 1999; Okuse et al., 1997; Shang & Dudley, 2005). The regulation of mRNA stability, cellular and subcellular localisation and translation are also mediated by known Nav splice variants (S. D. Dib-Hajj, Tyrrell, & Waxman, 2002; Shang & Dudley, 2005). MicroRNAs may also influence post-transcriptional/pre-translational Nav mRNA regulation (Bushati & Cohen, 2007). In addition, post-translational modifications and intracellular trafficking take place during the maturation and functional expression of the sodium channel (Stocker & Bennett, 2006; Zhou et al., 2002). For example, aldosterone does not alter mRNA transcription or protein expression levels of Nav1.5 but acts to promote protein membrane externalisation (Boixel et al., 2006). The implications of this high degree of regulation are exemplified during

DRG neuronal development, where the transient expression of sodium channels, namely Nav1.3, is thought to mediate spontaneous activity observed during late-stage embryogenesis (Benn, Costigan, Tate, Fitzgerald, & Woolf, 2001). Furthermore, the reciprocal later-stage developmental down-regulation of Nav1.3, alongside an up-regulation of Nav1.8 and Nav1.9 suggests convergence of regulatory mechanisms and the presence of a highly coordinated process (Benn et al., 2001; Black, Langworthy, Hinson, Dib-Hajj, & Waxman, 1997).

The expression of Navs is also significantly altered during disease and injury. Following axotomy and spinal cord injury a down-regulation of tetrodotoxin-resistant (TTX-R) sodium channels (Nav1.8 and Nav1.9) occurs (Benn et al., 2001; Decosterd, Ji, Abdi, Tate, & Woolf, 2002; S. D. Dib-Hajj et al., 1999; S. D. Dib-Hajj, Tyrrell, Black, & Waxman, 1998). In contrast, Nav1.3 is up-regulated in peripheral sensory neurones following axotomy and chronic constriction injury (CCI) of the sciatic nerve (Black et al., 1999; S. D. Dib-Hajj et al., 1999; Waxman, Kocsis, & Black, 1994). Although highly expressed during embryonic development (Felts, Yokoyama, Dib-Hajj, Black, & Waxman, 1997), Nav1.3 is almost completely absent in adult neurones (Fukuoka et al., 2008; Waxman et al., 1994). As such, the up-regulation of Nav1.3 during spinal cord injury, which results in neuronal hyperexcitability, is thought to be a key driver in the development of neuropathic pain (Waxman & Hains, 2006). Changes are also observed after inflammatory insult, where an up-regulation of both Nav1.3 and Nav1.7 occurs after a few days and, in contrast to neuropathic pain models, Nav1.8 is also up-regulated (Black, Liu, Tanaka, Cummins, & Waxman, 2004).

Changes in sodium channel isoform expression extend beyond direct neuronal insult and are also seen following experimental-induced diabetic neuropathy and in pathologies associated with non-excitabile cells (Craner, Klein, Renganathan, Black, & Waxman, 2002). For example, the degree of invasive metastasis in cancer cells correlates with the re-emergence of an embryonic neuronal phenotype and the up-regulation of sodium channel expression (Brackenbury, Djamgoz, & Isom, 2008). This is thought to enable cancer cells to utilise existing migratory cellular machinery and increase their invasiveness (Brackenbury et al., 2008). Specific changes in the expression levels of sodium channel isoforms during development or following injury, fine tune cellular excitability and form the complex networks required for tissue function (Rush, Cummins, & Waxman, 2007). When this coordinated expression patterning is dysregulated or hijacked by aberrant cellular processes during pathogenesis, then considerable morbidity and disruption to normal behaviour can ensue. Of the primarily peripherally expressed sodium channels, Nav1.9 is significantly associated with pain phenotypes in man. Alongside Nav1.9 channelopathies, expression in nociceptive sensory neurones and multiple roles in rodent pain behaviours have implicated the channel in the regulation of neuronal excitability and transduction of noxious stimuli. This is particularly true for inflammatory pain, where Nav1.9 plays a significant role (Amaya et al., 2006).

1.5.3 BIOPHYSICAL CHARACTERISTICS OF Nav1.9

The TTX-resistant Nav1.9 isoform is expressed primarily by small diameter neurones of the peripheral nervous system and is implicated in nociception (S. D. Dib-Hajj et al., 1998). The channel possesses unique biophysical

characteristics producing a large persistent sodium current, with slow activation and inactivation kinetics, and a hyperpolarised voltage-dependent activation, close to the resting membrane potential (Coste, Osorio, Padilla, Crest, & Delmas, 2004; Cummins et al., 1999; S. Dib-Hajj, Black, Cummins, & Waxman, 2002; Ostman, Nassar, Wood, & Baker, 2008). The slow kinetics of Nav1.9 activation likely precludes the channel from contributing to the action potential 'up-swing' (Ostman et al., 2008). Indeed the sub-threshold membrane potential activation of Nav1.9 current and wide activation/inactivation gating overlap characteristics, implicate the channel in the regulation of neuronal excitability and nerve terminal sensitivity (Baker, Chandra, Ding, Waxman, & Wood, 2003; Herzog, Cummins, & Waxman, 2001; Priest et al., 2005). Importantly, the channel is also required for inflammatory pain sensation (Amaya et al., 2006; Priest et al., 2005).

Combinations of inflammatory mediators such as bradykinin, ATP, histamine, prostaglandin E₂ and noradrenaline are capable of potentiating the Nav1.9 current, which can result in moderate depolarisation of DRG neurones and increased excitability (Maingret et al., 2008). Indeed, individual mediators including ATP, PGE₂ and TNF- α have been shown to result in Nav1.9 Na⁺ current up-regulation (Baker, 2005; X. Chen et al., 2011; Rush & Waxman, 2004). From the literature it is currently unclear as to whether a singly applied inflammatory mediator is an adequate stimulus to evoke such current potentiation or whether a combined inflammatory soup is required (Maingret et al., 2008). What is clear is the link between inflammatory pathways evoking protein kinase C and G protein-dependent signal transduction and an increase in Nav1.9 Na⁺ current

(Baker, 2005; Baker et al., 2003; Maingret et al., 2008; Rush & Waxman, 2004). For example, when GTP γ S, a hydrolysis-resistant GTP analogue, is included in the internal pipette solution during patch-clamp recordings, Nav1.9 current is significantly potentiated (Baker et al., 2003; Maingret et al., 2008). Specifically, intracellular GTP γ S causes an approximate twofold greater current density after whole-cell rupture and dialysis for 15-20 minutes (Maingret et al., 2008; Vanoye, Kunic, Ehring, & George, 2013). The accompanying voltage dependence of activation is unchanged in GTP γ S-stimulated human Nav1.9, however a +10mV shift in voltage dependence of inactivation was observed (Vanoye et al., 2013). In murine Nav1.9 currents, a hyperpolarising shift in activation voltage dependence was seen following both GTP γ S and PGE₂ stimulation (Maingret et al., 2008; Rush & Waxman, 2004). These disparities are likely species, agonist, or cell-type dependent. Single-channel analysis of Nav1.9 in the presence of GTP γ S shows that potentiation of the Na⁺ current is mediated, not by an increase in channel permeation, but by an increase (~2.7x) in channel open probability and mean open time (Vanoye et al., 2013). This mechanism of Nav1.9 current up-regulation (as opposed to increased expression or changes in tempero-spatial distribution) explains the larger peak and persistent current typical after relatively acute exposure (~10mins) to exogenous inflammatory mediators. This is significant as potentiated steady-state Nav1.9 currents are purported to be large enough to depolarise the membrane potential and induce action potential firing (Baker et al., 2003; Baker & Wood, 2001).

1.5.4 ROLE OF Nav1.9 IN BEHAVIOURAL PAIN PHENOTYPES

The regulation of sensory neurone excitability mediated by Nav1.9 currents significantly impacts behaviour pain phenotypes in rodent models. Indeed, behavioural pain experiments have been conducted on multiple transgenic knock-out Nav1.9 mice or following knock-down of Nav1.9 to assess the contribution of Nav1.9 to nociception. The majority of studies have focused on somatic mechanical, thermal or inflammatory paradigms, as well as neuropathic models. In line with changes observed to the Nav1.9 Na⁺ current following stimulation by inflammatory mediators, these studies provide evidence for the involvement of Nav1.9 predominantly in inflammatory mechanical hypersensitivity, whilst showing no obvious role in neuropathic pain.

Pain hypersensitivity (measured through paw mechanical thresholds to von Frey hair probing and 50°C hotplate response latency) elicited to intraplantar administration of prostaglandin E₂, bradykinin, interleukin-1 β , capsaicin, P2X₃ and P2Y receptor agonists was reduced or absent in Nav1.9 -/- mice (Amaya et al., 2006). However, Nav1.9 does not contribute to mechanical and thermal basal pain thresholds or to mechanical hypersensitivity after nerve injury; with nerve growth factor (NGF: a known mediator in neuropathic pain) still able to elicit distinct hypersensitivity even in Nav1.9 -/- mice (Amaya et al., 2006; Priest et al., 2005). Nav1.9 is also required for thermal hypersensitivity and spontaneous behaviours after peripheral inflammation. When the general inflammatory agent, complete Freund adjuvant (CFA), is injected into the hindpaw, the induced thermal hypersensitivity is absent in Nav1.9 -/- animals, whilst in contrast to injection of individual mediators, a mechanical

hypersensitivity was observed (Amaya et al., 2006; Priest et al., 2005). This suggests that some stimuli may rely on alternative pathways independent of Nav1.9. Using a model of sub-acute and chronic inflammation (intraplantar carrageenan and CFA-induced monoarthritis, respectively), Nav1.9 proved to be an effector of both thermal and mechanical hypersensitivity (Lolignier et al., 2011). Furthermore, Nav1.9 intrathecal antisense treatment and intraplantar carrageenan injection in rats also induced reductions in thermal and pressure pain thresholds (Lolignier et al., 2011). Leo *et al.*, found that Nav1.9 contributed to the 2nd phase of formalin-induced pain behaviours (Leo, D'Hooge, & Meert, 2010). However, they failed to show any meaningful differences in carrageenan and CFA-induced pain behaviours in their model (Leo et al., 2010).

Nav1.9 has little impact on neuropathic pain models (in both spared nerve injury model and partial sciatic nerve injury model) and changes in mechanical thresholds were the same irrespective of genotype (Amaya et al., 2006).

However, Priest *et al.* reported altered thermal hypersensitivity and Leo *et al.* also described a decreased sensitivity to cold stimulus under neuropathic pain conditions (Leo et al., 2010; Priest et al., 2005). In rat antisense

oligodeoxynucleotides Nav1.9 knock-down studies, there appeared to be no effect on nerve injury-induced behavioural responses (Porreca et al., 1999).

Collectively these data show that Nav1.9 is involved in the generation of inflammatory, but not neuropathic, hypersensitivity although the exact stimulus and knock-out line used seems to have an impact on the phenotypic manifestation.

1.5.5 *Nav1.9* IN VISCERAL NEURONES AND BEHAVIOURAL STUDIES

In visceral pain studies, the role for *Nav1.9* remains controversial. Behaviour models seem to suggest a robust visceral phenotype post-inflammation, with a lack of hypersensitivity to colorectal or bladder distension observed in *Nav1.9* knock-out mice (Martinez & Melgar, 2008; Ritter, Martin, & Thorneloe, 2009). This is confirmed by expression studies showing the presence of *Nav1.9* mRNA transcripts in colon-projecting DRG neurones and *Nav1.9* immunoreactivity in the majority of colon-projecting sensory neurones (Hillsley et al., 2006; King, Macleod, & Vanner, 2009). However, persistent TTX-R Na^+ currents have been challenging to record in visceral back-labelled DRG neurones, with proportions reported at 0-13% (Beyak, Ramji, Krol, Kawaja, & Vanner, 2004; Gold, Zhang, Wrigley, & Traub, 2002). Further, *Nav1.9* has not been associated with neuronal hyperexcitability induced by chronic colonic inflammation (Beyak et al., 2004; Hillsley et al., 2006).

In an *in vivo* colorectal distension (CRD) model of visceral pain, normal pain responses were comparable between *Nav1.9* $+/+$ and *Nav1.9* $-/-$ animals (Martinez & Melgar, 2008). After intracolonic instillation of an acute inflammatory stimulus (toll-like receptor 7 activator, R-484), responses to phasic CRD were increased by 62% in wild-type animals, however in *Nav1.9* $-/-$ animals these responses remained unchanged. The animals still mounted an inflammatory response against R-484, with cytokine levels and macroscopic histopathology similar irrespective of genotype (Martinez & Melgar, 2008). In a study of pelvic innervation, this time in bladder afferents, again no difference in basal urodynamics was observed in *Nav1.9* $-/-$ animals. However, following

intravesicular administration of PGE₂, pelvic afferent recordings showed a sensitisation in the wild-type animal that was not observed in Nav1.9 knock-out mice. In addition, *in vivo* treatment with cyclophosphamide did not evoke a reduction in bladder capacity in Nav1.9 -/- animals but did in Nav1.9 +/+ mice, again implicating Nav1.9 in pelvic afferent sensitisation to inflammatory mediators (Ritter et al., 2009). By contrast, intraperitoneal acetic acid injection in a 'writhing response' model of visceral pain caused a doubling in recorded writhes in Nav1.9 -/- mice compared to controls over a 15 minute period (Leo et al., 2010).

There have been two studies examining chronic colonic inflammation and the role of Nav1.9 in colon-projecting DRG neurone hyperexcitability. In a resolved post-infectious inflammatory model, back-labelled spinal neurones from Nav1.9 -/- and Nav1.8 -/- animals were compared to wild-type animals. 20-24 days post-*Nippostrongylus brasiliensis* (Nb)-induced jejunitis, isolated DRG neurones exhibited marked hyperexcitability, as measured by the number of action potentials elicited at 2x rheobase. This excitability was absent in Nav1.8 -/- mice but unchanged in Nav1.9 -/- animals, suggesting that Nav1.8, but not Nav1.9, is involved in regulating visceral neuronal hyperexcitability post-infection (Hillsley et al., 2006). It is likely that the whole-cell current-clamp paradigm used may bias the study towards observing changes in action potential number at steady-state plateau during current injection. As such, with a 2x rheobase current injection, Nav1.9's function at sub-threshold levels and for self-sustaining plateau depolarisations will be circumvented perhaps

explaining the lack of genotype-specific changes observed (Maingret et al., 2008).

Using colon retrograde-labelled DRG neurones from trinitrobenzenesulfonic acid (TNBS)-treated and control mice, Beyak *et al.* examined acute chronic visceral hyperexcitability (Beyak et al., 2004). TNBS-induced colitis produced a thickening of the colon and ulceration, which was associated with a significant hyperexcitability in small DRG neurones innervating the colon. Of the mouse colonic DRG neurones examined, < 15% expressed a persistent TTX-R I_{Nav} consistent with that produced by Nav1.9 (Beyak et al., 2004). In this model, the authors found no obvious difference in the number of neurones expressing Nav1.9 currents or their magnitude between control and TNBS-treated animals (Beyak et al., 2004). It is difficult, however, to draw conclusions from this study as the number of cells where a persistent TTX-R I_{Nav} were recorded are low ($N = 2$ and $N = 4$ from Nav1.9 +/+ and Nav1.9 -/- animals, respectively), suggesting that a more detailed study specifically targeting Nav1.9 in colonic DRG neurones is required. Indeed, methodological considerations around recording solutions, functional channel states and the rapid disappearance of the current, may all contribute to the variability in demonstrating the presence of Nav1.9 currents by patch-clamp electrophysiology (Baker et al., 2003; Cummins, Sheets, & Waxman, 2007; Leffler, Herzog, Dib-Hajj, Waxman, & Cummins, 2005).

In summary these data suggest that Nav1.9 is involved in afferent hypersensitivity to a variety of inflammatory stimuli. Behavioural models also implicate Nav1.9 in visceral afferent hypersensitivity following exposure to inflammatory stimuli, although this has yet to be shown conclusively with the

experimental paradigms currently employed. As a consequence the contribution of Nav1.9 to visceral afferent sensitivity remains to be defined.

1.6 NOXIOUS STIMULATION OF COLONIC VISCERAL AFFERENTS

In order to assess the role of Nav1.9 in visceral afferent function, relevant stimuli exposed to GI tract nerve termini during pathology must be considered. Such stimuli will likely participate in the pathogenesis of visceral pain associated with IBD also. These stimuli can be split into four broad categories. Firstly, direct noxious mechanical stimuli, such as that caused by distension or occlusion of the bowel. Mechanical stimuli will directly activate ion channels at the nerve ending and evoke action potential firing. Secondly, neurotransmitters, such as ATP, released into the nerve terminal microenvironment by mechanical stimuli, will excite and potentially sensitise afferent fibres. Thirdly, the effect of acute inflammatory mediators on afferent excitability and sensitivity must be assessed. Finally, chronic inflammation and a potentially differing set of inflammatory mediators will also impact visceral afferent function. Each of these is addressed in more detail below and the role Nav1.9 plays in modulating responses evoked by these stimuli is studied in subsequent chapters.

1.6.1 DIRECT NOXIOUS STIMULI – MECHANOSENSATION AND MECHANOTRANSDUCTION

The molecular transduction of mechanical stimuli by visceral afferents is central to the ability to convey gastrointestinal sensations and, in particular, is the primary cause of pain. Numerous molecular entities have been implicated in mechanotransduction in sensory neurones, some with apparent preferential

expression and function in visceral afferents. Mechanical stimuli can be transduced directly by mechanical modalities (e.g. compression, distension, etc.) opening mechanically-gated ion channels. These mechanically-gated channels are pore-forming and open very rapidly ($< 5\text{ms}$) (Brierley, 2010). Alternatively, mechanically-sensitive channels may trigger intracellular signalling pathways leading to the activation of downstream ion channels. The ability for a mechanical stimulus to evoke action potential firing from an afferent ending is also dependent on neuronal sensitivity, which is dramatically affected by acute or chronic inflammation. Candidate mechanically-gated ion channels for visceral mechanosensation are detailed below, including Acid Sensing Ion Channels (ASIC) and Transient Receptor Potential (TRP) ion channels, as well as modulators of afferent sensitivity.

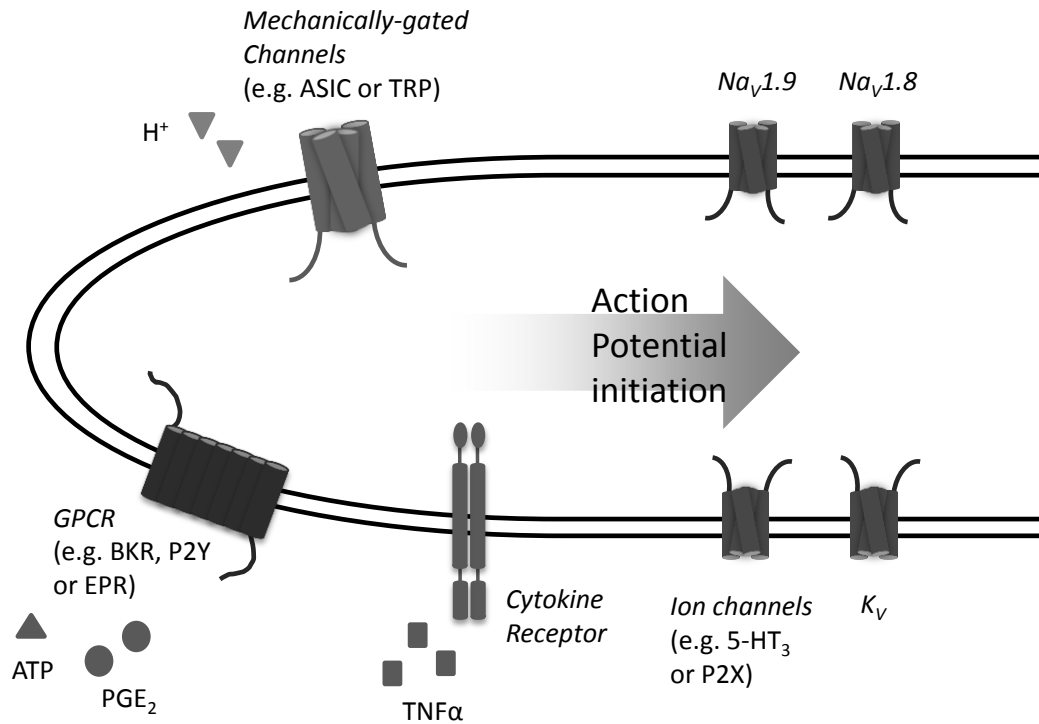


Figure 5. Receptor mechanisms on visceral afferent endings in the gastrointestinal tract. Mechanical stimuli are directly transduced through mechanically-gated channels (e.g. TRPV4, TRPA1, ASIC2) leading to depolarisation of the afferent ending. G-protein coupled receptors (GPCRs) and cytokine receptors for inflammatory mediators activate intracellular signalling pathways leading to both depolarisation and sensitisation of the ending, potentially through the post-translational modification of Na_v , K_v and/or non-selective ion channels (e.g. P2X, TRPV1). BK, bradykinin receptor; EPR, prostaglandin receptor; Na_v , voltage-gated sodium channel; K_v , voltage-gated potassium.

1.6.1.1 Acid Sensing Ion Channels (ASIC)

The degenerin/epithelial Na⁺ channel family of cation channels (DEG/ENaC) were first identified as mechanotransducers in *Caenorhabditis elegans* (M. Driscoll & Chalfie, 1991). In mammals, three members of the ENaC family, ASIC1, ASIC2 and ASIC3, have been linked to visceral mechanotransduction by their expression on mechanosensitive neurones and mechanosensory deficits in mutant null mice (Page, Brierley, et al., 2005). ASIC channels form pore-forming trimeric heteromultimers which are activated by protons and both mechanical and chemical stimuli.

ASIC1 is expressed in 30% of thoracolumbar afferents innervating the colon (Hughes, Brierley, Young, & Blackshaw, 2007) and ASIC1 knock-outs have a modest increase in the sensitivity of both vagal gastro-oesophageal mechanoreceptors and spinal splanchnic afferents (Page et al., 2004; Page, Brierley, et al., 2005). This is in contrast to cutaneous mechanoreceptors where sensitivity in ASIC1 null mice is unchanged (Page et al., 2004). ASIC2, however, does have a role in somatic mechanosensation with its loss leading to decreased sensitivity of slowly and rapidly adapting mechanoreceptors (Price et al., 2000). Approximately half of splanchnic DRG neurones and vagal gastro-oesophageal neurones express ASIC2 (Hughes et al., 2007; Page, Brierley, Martin, Hughes, & Blackshaw, 2007). Interestingly, loss of ASIC2 leads to opposing effects in these afferent populations, with an increase in the mechanosensory responses of serosal splanchnic afferents and a decrease in those of vagal IGLs or 'tension' receptors (Page et al., 2004; Page, Brierley, et al., 2005). By contrast, mesenteric splanchnic afferents were unaffected by deletion of ASIC2 (Page,

Brierley, et al., 2005). This suggests that molecular specialisation in mechanotransducers may exist between mesenteric and serosal afferents. In somatic models, ASIC3 knock-out reduces A δ fibre mechanosensitivity and increases rapidly adapting mechanoreceptor sensitivity (Price et al., 2001). However in the viscera, consistent deficits in mechanosensation are observed in vagal 'tension' receptors, mesenteric and serosal vascular splanchnic afferents and pelvic muscular/mucosal afferents (Bielefeldt & Davis, 2008; Page, Brierley, et al., 2005). This is in agreement with significant expression in almost three-quarters of splanchnic thoracolumbar DRG neurones (Hughes et al., 2007) and a significantly reduced visceromotor response to colorectal distension *in vivo* (Jones, Xu, & Gebhart, 2005). Therefore ASICs represent a significant contribution to specific aspects of both visceral and somatic mechanotransduction, and will likely influence the encoding of noxious mechanical stimuli.

1.6.1.2 Transient Receptor Potential (TRP) channels

TRP channels are non-selective cation channels consisting of six transmembrane domains and are transducers of mechanical, thermal and chemical stimuli. TRP channel activities are also significantly modulated by inflammatory mediators and contribute to afferent hypersensitivity following inflammatory insult. Of the 7 members of the TRP family (TRPA, TRPC, TRPM, TRPML, TRPN, TRPP and TRPV), a number have been associated with visceral mechanotransduction, including Transient Receptor Potential Vanilloid 1 (TRPV1), Transient Receptor Potential Vanilloid 4 (TRPV4), and Transient

Receptor Potential Ankyrin 1 (TRPA1) (Brierley et al., 2009; Brierley et al., 2008; Jones et al., 2005).

TRPV1 is expressed in over 80% of thoracolumbar DRG neurones and between 50-60% of lumbosacral colonic DRG neurones (Christianson et al., 2006; Robinson et al., 2004). Gastro-oesophageal, jejunal and pelvic colonic distension-sensitive afferents consistently show reduced mechanosensitivity in TRPV1 null mice (Bielefeldt & Davis, 2008; Jones et al., 2005; Rong et al., 2004). This contrasts with somatic mechanosensory function, where no difference is observed following deletion of TRPV1 (Caterina et al., 2000). Interestingly, TRPV1 is not thought to be mechanically gated and therefore may influence mechanosensation by indirectly affecting neuronal excitability or via interactions with other TRP channels, ion channels or receptors.

TRPV4 appears to have a more direct role in visceral mechanotransduction, with greater TRPV4 mRNA expression in visceral-projecting neuronal populations compared to the whole DRG population (Brierley et al., 2008). Colonic serosal and mesenteric afferent mechanosensory responses were also reduced by deletion of TRPV4 or by the application of an TRP antagonist (Brierley et al., 2008). However, pelvic mucosal, muscular and muscular/mucosal afferents, as well as vagal gastro-oesophageal mechanoreceptors, were unaffected by loss of TRPV4. The specific deficits observed in these knock-out mice suggest that TRPV4 may play a specialised role in the mechanotransduction of high-threshold nociceptor-like afferents in the colon (Brierley et al., 2008). These reductions in mechanosensory function are also apparent during *in vivo* visceromotor responses to colorectal distension

in both TRPV4 null mice and following small interfering RNA (siRNA) knock-down of TRPV4 also in mouse (Brierley et al., 2008; Cenac et al., 2008).

TRPA1 represents another member of the TRP family required for visceral mechanotransduction. Specifically TRPA1 contributes to mechanotransduction in serosal, mesenteric and mucosal afferents but not those afferents responsive to stretch (Brierley et al., 2009). TRPA1 is expressed by just over half of gastro-oesophageal, splanchnic colonic and pelvic colonic DRG neurones (Brierley et al., 2009). Decreased visceral motor reflex (VMR) to CRD in TRPA1 null mice was also confirmed in rat by intrathecal injection of TRPA1 antisense oligodeoxynucleotide (Brierley et al., 2009; J. Yang et al., 2008). In conjunction with TRPA1's involvement in normosensation, it also appears to play a central role in bradykinin-induced mechanical hypersensitivity in both guinea-pig oesophagus and mouse colon. However, direct chemosensory responses to bradykinin are unchanged suggesting a sensitising role for TRPA1 (Brierley et al., 2009; S. Yu & Ouyang, 2009).

Other TRP channels that have been implicated in mechanotransduction include the cold-sensing TRPM8 channel, as well as TRPN1 and TRPC3/C6 (Harrington et al., 2011; Quick et al., 2012; Vollrath, Kwan, & Corey, 2007). Further studies, including those in knock-out mice, are required to fully understand the mechanosensory functions of this diverse family of channels in visceral afferents.

1.6.1.3 Other putative mechanotransducers

The recently identified mechanically activated Piezo family of cation channels may also prove to be relevant to visceral mechanosensory function. This new class of membrane protein contain 24-36 transmembrane segments, with a similar structure to voltage-gated sodium channels, are described as transducers for rapidly-adapting mechanosensitivity in DRG neurones (Coste et al., 2010). However, it remains to be seen whether Piezo null mice will possess deficits in visceral mechanosensory function.

How Nav1.9 impacts on visceral afferent function during mechanical stimulation remains poorly understood. Normosensation appears largely unaffected during somatic behavioural models in Nav1.9 ^{-/-} mice, suggesting that the channel may not be critical to the direct transduction of mechanosensation (Amaya et al., 2006; Priest et al., 2005). Utilising multiple mechanical stimuli, including ramp distension, phasic distension and von Frey hair probing of the colorectum, will facilitate a more comprehensive understanding of Nav1.9 in visceral mechanotransduction.

1.6.2 STIMULUS RELEASED MEDIATORS - ROLE OF PURINERGIC SIGNALLING IN VISCERAL AFFERENT FUNCTION

Visceral hypersensitivity in response to balloon distension of the colorectum correlates with severe pain in irritable bowel syndrome patients (Kuiken, Lindeboom, Tytgat, & Boeckxstaens, 2005). One algogenic mediator which has been shown to be particularly relevant to the activation of visceral nociceptors during distension is ATP. George Burnstock proposed that in hollow visceral organs, e.g. tubes (intestines, ureter, bile duct, salivary duct) and sacs (lung,

urinary bladder, gall bladder), mechanical stress, and therefore distension, can trigger the release of ATP from the epithelial lining leading to the stimulation of visceral nociceptors (Burnstock, 1999, 2009a). In addition, release of ATP by colorectal distension is greatly enhanced during colitis suggesting a more prominent role for this pathway during inflammation (Calvert, Thompson, & Burnstock, 2008; Shinoda, Feng, & Gebhart, 2009; Wynn, Ma, Ruan, & Burnstock, 2004). With regards to purinergic signalling, application of ATP has been shown to enhance Nav1.9 currents, and the mechanical hypersensitivity to intraplantar injections of purinergic ligands (α,β -methylATP and UTP) is lost in Nav1.9 knock-out mice (Amaya et al., 2006; Baker, 2005). Given the importance of ATP as a signalling molecule for visceral pain, it is likely that Nav1.9 may represent a novel transduction mechanism of visceral afferent sensitisation for purinergic stimuli.

1.6.3 ACUTE INFLAMMATORY MEDIATORS – EFFECT OF EXPERIMENTAL INFLAMMATORY SOUP

During tissue damage and inflammation, multiple mediators are released that act to sensitise afferent terminals (Grundy, 2004; Su & Gebhart, 1998). Indeed, recurrent bouts of acute mucosal inflammation will undoubtedly repeatedly excite and sensitise visceral nerve termini in IBD patients. The mediators responsible may include bradykinin, ATP, PGE₂, histamine and 5-HT, which are released by damaged or stressed cells, as well as the immune and vascular systems. Together these act to hypersensitise afferents to mechanical stimuli and drive direct stimulation of afferent endings (Grundy, 2004). Experimental inflammatory soups, consisting of a combination of inflammatory mediators, have been used to study changes in visceral afferent sensitivity to inflammatory

stimuli. Indeed inflammatory soup applied intracolonicly increases resting afferent activity and the magnitude of firing in rat (Su & Gebhart, 1998).

Inflammatory soups have also been used to investigate mechanically insensitive afferents by sensitising otherwise 'silent' fibres (Feng & Gebhart, 2011). The functional up-regulation of Nav1.9 current amplitudes by inflammatory pathways suggest that Nav1.9 will significantly contribute to afferent excitability following acute stimulation by inflammatory mediators (Baker, 2005; Maingret et al., 2008).

1.6.4 CHRONIC INFLAMMATORY MEDIATORS - EFFECT OF IBD ON VISCERAL AFFERENT FUNCTION AND LIKELY ROLE FOR Nav1.9

In addition to acute inflammatory phases, advanced late-stage IBD represents a disease paradigm with patients suffering from chronic inflammation and chronic pain. A central component of the pain experienced in IBD is so called IBS-associated post-inflammatory hypersensitivity (Grover et al., 2009).

Experimentally, visceral hypersensitivity in IBS is observed to colorectal distension (Lembo et al., 1994; Ritchie, 1973) and is likely the result of long-term mechanistic changes in sensory nerve terminal sensitisation by inflammatory mediators. Using visceral rodent models, supernatants derived from inflamed and normal human bowel tissues have been used to study the impact of human-derived inflammatory milieu on visceral afferent modulation in an acute setting. Faecal supernatants from diarrhoea-predominant IBS patients when instilled into the colorectum of mice induce colonic hypersensitivity (Annaházi et al., 2009). Indeed, visceral hyperalgesia is also evoked by supernatants derived from colonic mucosal biopsies of IBS patients

(Cenac et al., 2007). Similar disease supernatants, but not normal controls, are capable of increasing nerve discharge from rat mesenteric afferents (Barbara et al., 2007), suggesting that an inflammatory milieu released by the mucosal lining is capable of exciting and sensitising visceral afferents transducing pain. IBS-like symptoms, including diarrhoea and abdominal pain, disproportionate to the degree of active inflammation, may be experienced by IBD patients (Grover et al., 2009; Isgar, Harman, Kaye, & Whorwell, 1983; Minderhoud, Oldenburg, Wismeijer, van Berge Henegouwen, & Smout, 2004). By contrast, a subset of IBD patients, in a remissive and quiescent state, appear normosensitive or even hyposensitive to colorectal distension (Bernstein et al., 1996; Chang et al., 2000). This has been reinforced by ulcerative colitis faecal supernatants causing colonic hyposensitivity when infused in the colorectum of mice (Annaházi et al., 2009). Differences in the sensitivity of IBD patients to distension and the apparent ability for IBD supernatants to evoke hyposensitivity in mice likely reflect the spectrum of disease progression and severities encompassed by 'inflammatory bowel disease'. Consequently, the inflammatory environment that the nerve terminal is exposed to is also likely to change significantly over the course of disease progression.

These changes in visceral afferent function have been associated with multiple inflammatory mediators, including those released by mast cells, such as histamine, tryptase and trypsin (Annaházi et al., 2009; Barbara et al., 2007; Cenac et al., 2007). It is likely that the combination of inflammatory mediators released by the chronically damaged mucosal lining of the gut during IBD differs significantly from those released during more acute inflammation. Whilst the

specifics of the mechanism leading to changes in visceral sensitivity is unknown, differing combinations of inflammatory mediators acting at ion channels and GPCRs will likely lead to visceral afferent modulation. Given that Nav1.9 contributes significantly to the regulation of afferent sensitivity suggests that Nav1.9 may play an important role in the development of visceral pain during conditions such as inflammatory bowel disease.

1.7 PRINCIPAL AIM

To investigate the role of Nav1.9 in visceral afferent activation to noxious inflammatory, mechanical and human-disease derived stimuli, relevant to inflammatory bowel disease.

CHAPTER 2: LOCALISATION OF $\text{Na}_v1.9$ IN VISCERAL AFFERENT FIBRES

2.1 INTRODUCTION

2.1.1 *EXPRESSION OF $Na_v1.9$ IN MAMMALIAN TISSUES*

The tetrodotoxin-resistant (TTX-R) $Na_v1.9$ isoform is primarily expressed within neurones of the peripheral nervous system (PNS), however low level expression is observed in the CNS. In mouse, $Na_v1.9$ was identified by quantitative RT-PCR in both brain and spinal cord, whilst in guinea pig, a very low level of expression to no expression, was seen in brain tissues (Hillsley et al., 2006; Kwong et al., 2008). In adult rat, $Na_v1.9$ protein is expressed in areas containing central projections of sensory neurones (e.g. dorsal horn of the spinal cord) by immunohistochemistry (IHC) (Amaya et al., 2000; S. Dib-Hajj et al., 2002), however mRNA transcripts were not observed in spinal cord samples (S. D. Dib-Hajj et al., 1998). Furthermore, expression by RT-PCR showed $Na_v1.9$ mRNA expression in cerebral hemispheres but not the cerebellum, suggesting specific regional distributions (S. D. Dib-Hajj et al., 1998). Perhaps the most convincing evidence for functional expression of $Na_v1.9$ within the CNS is the inhibition of brain derived neurotrophic factor (BDNF)-evoked sodium currents in rat hippocampal neurones by antisense $Na_v1.9$ treatment (Blum, Kafitz, & Konnerth, 2002). Importantly, no evidence has been found for $Na_v1.9$ expression in any other naïve adult excitable cell types or mammalian tissues. These include skeletal and cardiac muscle, spleen, lung, liver, kidney, uterus, testis and adrenal gland, following investigation by quantitative RT-PCR, Northern blot and *in situ* hybridisation (ISH) (S. D. Dib-Hajj et al., 1998; Kwong et al., 2008).

In the peripheral nervous system (PNS), Nav1.9 expression is restricted to the cell bodies and projections of sensory neurones and the enteric nervous system. Interestingly, given the contrasting effects of Nav1.7 mutant expression in dorsal root ganglia (DRG) versus sympathetic neurones on neuronal excitability, it is of relevance that sympathetic neurones do not appear to express Nav1.9 (S. D. Dib-Hajj et al., 1998; Rush et al., 2006). In the dorsal root, nodose and trigeminal ganglia, Nav1.9 is expressed by the vast majority of small diameter neuronal cell bodies and in their central terminal arborisations in dorsal horn lamina II of the spinal cord as well as the spinal trigeminal nucleus in the medulla (Amaya et al., 2000; S. D. Dib-Hajj et al., 1998; Kwong et al., 2008). There is also evidence for Nav1.9 in extrinsic primary afferent innervation of the viscera, with significant expression within both the vagal innervation and gut-projecting sensory DRG neurones (King et al., 2009; Kwong et al., 2008). Expression of Nav1.9 in peripheral projections has been identified by immunohistochemistry (IHC) in fibres innervating the skin (S. Dib-Hajj et al., 2002; Liu et al., 2001; Lolignier et al., 2011; Padilla et al., 2007), cornea (Fjell et al., 2000) and molar (Padilla et al., 2007). In addition, both unmyelinated and myelinated teased sciatic nerves (S. Dib-Hajj et al., 2002; Fjell et al., 2000; Liu et al., 2001) and lung-specific nodose neurones (Kwong et al., 2008) also exhibit specific expression of Nav1.9. Analysis by IHC of the enteric nervous system shows Nav1.9 expression in myenteric and submucosal enteric neurones in mouse colon (Padilla et al., 2007; Rugiero et al., 2003); specifically interplexus fibres and axonal projections passing through all layers of the bowel wall (longitudinal muscle, myenteric plexus, circular muscle, submucosal plexus and mucosa) (Padilla et al., 2007).

2.1.2 EXPRESSION OF *Nav1.9* IN SENSORY NEURONES

Although *Nav1.9* is found to some extent in other mammalian tissues, the expression in approximately 82% of small DRG neurones has focused research to this predominantly nociceptive phenotype (S. D. Dib-Hajj et al., 1998; Fukuoka et al., 2008). *Nav1.9* expression by *in situ* hybridisation (ISH) and IHC is found in approximately 40-70% of DRG neurones (see Table 3) with considerably higher levels in small neurones (diameter <30µm: 81.6-82.9%) and significantly lower levels are observed in larger neurones (≥30µm: 18.8-27.4%) (S. D. Dib-Hajj et al., 1998; Fukuoka et al., 2008). Whilst IHC and ISH techniques suggest a relatively high degree of expression, functionally the recording of *Nav1.9* currents by patch-clamp suggests there are a smaller proportion of neurones expressing *Nav1.9*. For example, Baker *et al.* report 1 in 6 small neurones possess *Nav1.9* currents (Baker, 2005; Baker et al., 2003). Cummins *et al.* suggest approximately 60% of small *Nav1.8*-null neurones develop persistent sodium currents consistent with that mediated by *Nav1.9* (Cummins et al., 1999). Within the colon-projecting DRG neuronal population this disparity is far greater, with persistent TTX-R Na⁺ currents, attributed to *Nav1.9*, recorded from between 0-13% of cells (Beyak et al., 2004; Gold et al., 2002).

Importantly, the presence of *Nav1.9* mRNA and protein has been detected, but yet not quantified, in retrogradely-labelled neurones from the colon, suggesting the channel is expressed (Hillsley et al., 2006; King et al., 2009). In agreement with this, *Nav1.9* ^{-/-} mice fail to develop post-inflammatory visceral hypersensitivity; again implicating *Nav1.9* in visceral projecting neuronal

function (Martinez & Melgar, 2008; Ritter et al., 2009). As such, quantifying the extent of Nav1.9 expression within colonic DRG neurones will help to understand why persistent Na⁺ currents were recorded at such low frequency in this population.

Na _v 1.9 Expression levels in DRG neurones (% of)			Comments	References
Diameter				
<i>Total</i>	<i><30μm</i>	<i>≥30μm</i>		
<i>By in situ hybridisation</i>				
58.8 -61.5	81.6	27.4	Rat DRG	(Fukuoka et al., 2008)
69.5	82.9	18.8	Rat DRG	(S. D. Dib-Hajj et al., 1998)
<i>By immunohistochemistry</i>				
38	-	-	Naïve rat DRG	(Decosterd et al., 2002)
41	-	-	Adult rat DRG	(Benn et al., 2001)
-	56	-	Dissociated rat DRG neurones	(Fjell et al., 2000)

Table 3. Expression of Na_v1.9 in DRG neurones

Interestingly, Nav1.9 is not found in all nociceptive neurones (64% of C-fibre nociceptors and 54% of A δ -fibre nociceptors), but where it is expressed it does contribute to membrane properties consistent with a nociceptive phenotype (Fang et al., 2002; Tate et al., 1998). Where functional identification (such as firing in response to noxious stimuli) of a nociceptive phenotype is not possible, protein markers are often used to further characterise neurones and identify nociceptive populations. For example, expression of neurofilament heavy (NF200) and peripherin can differentiate between large myelinated A-fibre and small unmyelinated C-fibre neurones, respectively (Goldstein, House, & Gainer, 1991; Lawson & Waddell, 1991). C-fibres can be further segregated into two broad, likely overlapping populations: those non-peptidergic neurones that bind isolectin-B4 (IB4) from the plant *Griffonia simplicifolia*, and those peptidergic neurones that express calcitonin-regulated gene product (CGRP) and/or substance P (SP) (Hunt & Mantyh, 2001). Importantly these two groups terminate in different layers of the dorsal horn: lamina I-II(outer) for peptidergic and lamina II(inner) for non-peptidergic (Hunt & Rossi, 1985). Whilst both groups of neurones respond to noxious stimuli and express the TRPV1 receptor, a known noxious thermal and mechanical transducer, they do differ in their expression of other nociceptive markers (Hunt & Mantyh, 2001). In addition to binding IB4, non-peptidergic neurones also largely express P2X₃ and receptors for glial cell line-derived neurotrophic factor (GDNF), whilst peptidergic populations express the nerve growth factor (NGF) receptor trkA. Importantly, both trkA expression and IB4 binding have been directly associated with a nociceptive phenotype *in vivo* (Fang, Djouhri, et al., 2005; Fang et al., 2006). Binding of IB4 to α -D-galactose residues on the extracellular

membrane of sensory neurones is shown to correlate exclusively with unmyelinated C-fibre nociceptors (Fang et al., 2006; Gerke & Plenderleith, 2001) and strong trkA expression is observed in DRG nociceptors (Fang, Djouhri, et al., 2005).

There is significant colocalisation of Nav1.9 with the small unmyelinated C-fibre protein marker peripherin, in both DRG neurones and in peripheral projections to skin (S. Dib-Hajj et al., 2002; Lollignier et al., 2011). Practically all IB4 binding neurones also express Nav1.9 (>98% (Fukuoka et al., 2008)), though some neurones positive for expression of Nav1.9 (Nav1.9-positive; Nav1.9+) do not bind IB4 (60% - 73% IB4-positive of total Nav1.9-positive (Amaya et al., 2000; Benn et al., 2001; Fjell et al., 2000; Fukuoka et al., 2008)). This is consistent with co-expression of Nav1.9 and IB4 in central sensory neuronal projections in lamina II of the dorsal horn (S. Dib-Hajj et al., 2002).

Whilst Nav1.9 is expressed by the majority of IB4 binding non-peptidergic C-fibres, there is also a subset of trkA-positive, likely peptidergic, neurones in which it is expressed. ISH and IHC evidence suggest that ~45-70% of trkA-positive neurones express Nav1.9 (Amaya et al., 2000; Benn et al., 2001; Fukuoka et al., 2008). However, the intensity of Nav1.9 expression within a DRG neurone correlates with the intensity of IB4 binding but not trkA expression, consistent with regulation of Nav1.9 expression by GDNF, but not NGF (Fang, Djouhri, et al., 2005; Fang et al., 2006; Fjell et al., 1999).

Nav1.9 appears to have complex patterning within sensory neurones but what is clear is its primary expression in small C-fibre nociceptors classified as either peptidergic or non-peptidergic. Nav1.9 is strongly associated with IB4, yet is

also found in a subset of trkA-positive neurones. Remaining Nav1.9 expression can be explained by non-nociceptive expression in a minority of large slowly-conducting A-fibre neurones.

2.1.3 PROTEIN MARKER CHARACTERISATION OF VISCERAL SENSORY NEURONES

As outlined above, nociceptors are strongly associated with various biochemical and electrophysiological characteristics. In nearly all cases, this has focussed on stimulus-response functions to non-visceral innocuous and noxious sensations, including touch, pinch, heat, cut, crush and vibration. However, visceral afferents transduce unique sensations including bloating, distention, nausea and dyspnea, alongside pain. As such, visceral nociceptors are not required to encode sensations such as cutting or excessive heat, but are instead activated by obstructive, inflammatory, ischaemic or infiltrative processes (Knowles & Aziz, 2009). This has important implications for defining the visceral nociceptor, as nociceptors in the viscera are unlikely to represent a distinct and independent pathway that respond only to noxious stimuli (although mesenteric afferents may encode little other consciously perceived stimuli). Rather, the majority of visceral afferents innervating the gut are likely capable of encoding noxious, high intensity and normally painful stimuli. Alternatively, there is a lack of innocuous stimulus sensing A α / β fibre innervation in the viscera, with nearly all visceral afferents of C-fibre (60-80%) and A δ -fibre (20-40%) subtypes (Sengupta & Gebhart, 1994; Sengupta et al., 1990). By contrast almost 70% of cutaneous and muscle innervation from the sciatic nerve are A α / β -fibres, with C- and A δ fibres making up approximately 15% and 12%, respectively (Harper & Lawson, 1985a, 1985b). Within the lumbosacral pathway, a subset of visceral

afferents are specifically activated by high-threshold stimuli, and even those activated by low-threshold stimuli are intensity-encoding and respond through a range of innocuous to noxious stimuli (G. F. Gebhart, 2000). In the splanchnic nerve, almost all (80-90%) afferent fibres are of serosal and mesenteric subtype, which are thought to represent a major type of nociceptor (Brierley et al., 2004; Brookes et al., 2013). Furthermore, the significant ability for visceral afferents to sensitise following chemical stimulation by inflammatory mediators, with 'silent' nociceptors or mechanically insensitive afferents acquiring mechanosensitivity following inflammation, suggests pronounced plasticity in the sensitivity of visceral afferents, which likely underlies their concurrent function as nociceptors (Feng & Gebhart, 2011).

As such the application of known nociceptive protein markers to visceral afferents, in the absence of functionally defined visceral-nociceptor populations, are discussed below. In line with the focus of this thesis, the emphasis of discussion will be on spinal colonic innervation. Retrograde labelling studies of the rodent colon suggest that between 3 and 15% of DRG neurones project to the colon, with a bimodal distribution amongst spinal cord levels: one at spinal cord level T8-L1 in mouse (T8-L2 in rat) and the other comprising L6-S1 in mouse (L6 – S1 in rat) (Brierley et al., 2008; Christianson et al., 2006; Jänig & Morrison, 1986; Perry & Lawson, 1998; Robinson et al., 2004). The thoracolumbar pathway corresponds to afferent fibres tracking with the splanchnic nerve and the lumbosacral pathway that of afferent fibres tracking with the pelvic nerve.

Whilst A α / β -fibres are rare in viscerally projecting populations, neurofilament-heavy markers have proven useful in defining the thinly myelinated A δ -fibre visceral afferent population (between 19-26% of visceral afferents (Christianson et al., 2006; Perry & Lawson, 1998)). In contrast are non-visceral C-fibres, where there is a significant peptidergic bias in visceral afferents, such that around 80-81% of colon-projecting neurones express CGRP (Christianson et al., 2006; Robinson et al., 2004) and this rises to 99% when whole splanchnic nerve retrograde labelling is performed (Perry & Lawson, 1998). This is consistent with significant expression of CGRP in peripheral projections along vasculature of the intestinal mesentery and submucosa (Song et al., 2009). The vast majority of these fibres also express substance P (~80%) (Perry & Lawson, 1998). With that in mind, the binding of IB4 (purported correlate of non-peptidergic C-fibres) is markedly less than that observed in non-visceral afferents. In colonic projecting afferents this is as low as 5-20% (Christianson et al., 2006; Robinson et al., 2004). Therefore the protein markers IB4 and CGRP do not segregate visceral afferents into distinct groups as observed in non-visceral afferents, and in fact IB4-positive visceral afferents almost always additionally express CGRP (90-100% (Christianson et al., 2006; Robinson et al., 2004)).

Given the strong correlation between IB4 binding and Nav1.9 expression in cutaneous nociceptive neurones, it is necessary to characterise whether this correlation holds true in visceral afferents also (Fang et al., 2006). To address this and provide support for a functional role of Nav1.9 in visceral afferent sensitisation, *in situ* hybridisation and immunohistochemistry for Nav1.9 mRNA

and protein was performed on retrograde-labelled DRG neurones from the colon.

2.2 AIMS

1. The relatively low frequency of Nav1.9 persistent currents recorded by patch-clamp of colon projecting neurones is in contrast to both substantial behavioural phenotypes observed in Nav1.9 $-/-$ mice and existing qualitative expression data. As such, the first aim of this study is to determine the level of Nav1.9 expression within colon-projecting DRG neurones using both *in situ* hybridisation and immunohistochemical techniques.
2. There exists a strong correlation between IB4 binding and Nav1.9 expression in somatic nociceptors. However, IB4 expression is significantly lower in visceral sensory neurones and does not separate from peptidergic markers, such as CGRP, but rather colocalises with them. The second aim of this study is to further characterise the expression of Nav1.9 using the protein markers IB4 and CGRP, in comparison with known expression patterns in somatic projecting neurones.

2.3 METHODS

2.3.1 RETROGRADE LABELLING OF COLONIC TISSUES

Fast Blue (FB; 2% in saline, Polysciences GmbH, Germany) was injected into the wall of the distal colon of Nav1.9 $+/+$ and Nav1.9 $-/-$ mice and male Sprague-Dawley rats (150-250g). The Nav1.9 $-/-$ construct and breeding strategy for these animals is described in detail in Chapter 3 (3.3.1 Nav1.9 knock-out mice). Animals were anaesthetised with isoflurane then an approximate 1.5cm laparotomy performed. Five injections of 0.2 μ l FB per site, at a rate of 0.4 μ l/min via a microinfusion pump, were made into the wall of the distal colon using a fine-pulled glass needle. The muscle layer was sutured and the skin secured with Michel clips. Post-operative analgesia (buprenorphine 0.05-0.1mg/kg daily) and care (monitoring body weight and soft diet) was provided.

After three or seven days, animals were euthanised with sodium pentobarbital (200mg/kg i.p.) and transcardially perfused with saline (0.9%) followed by paraformaldehyde (PFA; 4% in 0.1M phosphate buffer; pH 7.4). Dorsal root ganglia (DRG; Nav1.9 $+/+$ and Nav1.9 $-/-$ mice, T7-L4; rats, T7-L4) were removed and post-fixed in 4% PFA for 2 hours and cryoprotected in 30% sucrose (w/v phosphate buffered saline (PBS)). Cryostat sections (10 μ m) were collected sequentially across 10 slides per DRG.

2.3.2 IMMUNOHISTOCHEMISTRY

Sections were blocked in antibody diluent (10% horse serum and 0.2% (v/v) Triton X-100 in 0.1M PBS) for 1 hour, followed by overnight incubation with primary antibodies (rabbit anti-Nav1.9 antibody (1:1000; Alomone, Israel) and

goat anti-CGRP antibody (1:2000; Abcam, UK)) and a 4 hour incubation with fluorophore-conjugated secondary antibodies (donkey anti-rabbit IgG-Alexafluor-488 and donkey anti-goat IgG-Alexafluor-568 (1:1000; Invitrogen, UK)) and/or isolectin B4 (IB4) from *Griffonia simplicifolia*-Alexafluor-647 (2.5µg/ml: Invitrogen, UK).

2.3.3 ISOTOPIC IN SITU HYBRIDISATION

Oligonucleotide probes complementary to bases 968-1001 (Probe 1) and 2641-2674 (Probe 2) of the mouse *SCN11A* sequence, accession number NM011887.3, were synthesised (Sigma, UK) and hybridized to DRG sections using previously described protocols, and were visualised by silver grain development in radiographic emulsion (Michael et al., 1997). Probes were labelled at the 3' end with ³⁵S-dATP (Perkin Elmer, UK) and terminal transferase (Promega, WI, USA). Equipment was cleaned using 1M NaOH and Diethylpyrocarbonate (DEPC)-treated H₂O to inactivate RNase enzymes. Sections were first acetylated in 0.25M acetic anhydride/0.1M triethanolamine (in DEPC Phosphate Buffered Saline (PBS)), passed through a dehydrating ethanol series (70-100%) and then delipidated with chloroform for 5 minutes. Hybridisations occurred overnight at 37°C using a probe concentration of 2nM. The probe was diluted in hybridisation buffer (2X Denhardts solution (Sigma, UK), 4X SSC (1X SSC = 150mM sodium chloride, 15mM sodium citrate, pH 7.0), 50% deionised formamide, 10% dextran sulphate (Pharmacia Biotech, UK), 100µg/ml Poly A⁺ (Sigma, UK), 100µg/ml sheared salmon sperm DNA (Boehringer, Germany), 20mM dithiothreitol (DTT)) and briefly (5 minutes) heated to 65°C. After hybridisation, sections were washed twice in 2x SSC

(containing 0.4% β -mercaptoethanol) at room temperature, then twice in 1X SSC at 50°C and once in 0.2X SSC at 50°C. Sections were washed for an additional hour in 1X SSC at room temperature, dehydrated with ethanols (70-100%) and allowed to air dry, before dipping in autoradiographic emulsion (K5 Nuclear Emulsion, Process Supplies, UK). Sections were exposed for 2-3 weeks, developed, counterstained with Giemsa counterstain and coverslipped. No specific labelling was observed in DRG sections of Nav1.9 $-/-$ mice hybridised with *SCN11A* probes; reactions where an excess of unlabelled probe was used resulted in only background signal.

2.3.4 ISOTOPIC IN SITU HYBRIDISATION PROBE SELECTION

34 base oligonucleotide probes complementary to bases 968-1001 (Probe 1), 2641-2674 (Probe 2) and 5041-5074 (Probe 3) of the mouse *SCN11A* sequence, accession number NM 011887.3, were selected with 52.9% GC content.

Oligonucleotide probes were checked for self-dimerisation and hairpin formation, alongside homology to other mRNA sequences (Kibbe, 2007).

Furthermore, homology to rat *SCN11A* sequence, accession number NM 019265.2, was also established (see Table 4), for potential utility in rat DRG sections.

	Complementary base sequence	GC %	Secondary structure	Homology to rat SCN11A
Probe 1	GAGGACTTCATAATGTGTGGCAACTGGCTCGGCA	52.9	None	91%
Probe 2	TGAGAGTAGAGACACAGCCACCCTGGATACAAGG	52.9	None	29%
Probe 3	GGATGTTCTCTTTGCTTTCACCACCAGGGTCCTC	52.9	None	97%

Table 4: *In situ* hybridisation probes against mouse SCN11A

2.3.5 CHROMOGENIC *IN SITU* HYBRIDISATION

Retrograde-labelled DRG sections were prepared as described for isotopic *in situ* hybridisation, target probes against *SCN11A* mRNA hybridised for two hours and expression visualised using RNAscope Brown Detection Kit (Advanced Cell Diagnostics, Inc., USA). The chromogenic *in situ* hybridisation assay uses a proprietary signal amplification system to specifically detect probes hybridised to the tissue. Presence of the target mRNA transcript is detected by punctate dots of chromogen precipitate (brown). Specifically, sections were washed (0.1M PBS), endogenous enzyme activity blocked (10 minutes; room temperature (RT)) and antigen retrieval performed (0-20 minutes; 40°C) before probe hybridisation. The *SCN11A* probe was designed by ACD (*SCN11A*-C1(catalogue #403531)) and targets NM_011887.3, 349-1283bp, with 6-10 short oligonucleotide pairs, covering approximately 50 bases of the target mRNA per oligonucleotide. After hybridisation, multiple amplification steps were performed, culminating in the addition of horse radish peroxidase (HRP)-labelled probes. Addition of a chromogenic substrate produced the brown product identifying expression. Sections were counterstained (Giemsa) and coverslipped, before imaging.

2.3.6 *Nav1.9* ANTIBODY

The rabbit anti-Nav1.9 antibody used in these studies (Alomone ASC-017) was raised against an epitope on the intracellular C-terminus domain of the rat Nav1.9 channel (amino acid residues 1748-1765, accession number O88457). Although 83% sequence homology exists between mouse and rat Nav1.9 channels, significant non-specific labelling was present in pilot experiments in

mouse Nav1.9 ^{-/-} DRG sections. As such, the antibody was only used in rat tissues, where control experiments indicated specific labelling of Nav1.9 (see Figure 11).

2.3.7 IMAGING AND QUANTITATION

Relative intensities of reaction products in immunostaining for Nav1.9 and other markers and Nav1.9 mRNA *in situ* hybridisation were determined for all DRG cells with visible nuclei (ImageJ 1.45S analysis software, NIH, USA). The mean background absorbance of each section was subtracted prior to analysis to control for variability in illumination. Percentage relative intensities of background-subtracted cell absorbance were determined by comparison with least intensely (0%) and most intensely (100%) labelled profiles. Relative intensity of DRG cells was measured in Nav1.9 ^{-/-} sections to set the threshold (T_{pos}) for positive labelling in wild-type mice. Cells with intensity values greater than the mean intensity of the ten cells with the highest background intensity values in Nav1.9 ^{-/-} sections plus two times its standard deviation (SD), were considered positively labelled ($T_{\text{pos}} = 22\%$). For Nav1.9, CGRP and IB₄-like immunoreactivity, intensity of staining was scored on a scale of 0-5 by two independent observers, with 0 representing negative and 5 strongly positive. The mean absorbance of these cells taken from ImageJ analysis correlated significantly with staining scores (e.g. Pearson $r = 0.87$; $p < 0.0001$; $n = 149$) and a threshold for positive staining was determined as performed by Fang *et al.* (e.g. mean absorbance for Nav1.9 $\geq 32\%$) (Fang *et al.*, 2002). In mice neurones were classified as small ($<400\mu\text{m}^2$ (diameter $\sim <20\mu\text{m}$)) or large ($\geq 400\mu\text{m}^2$ (diameter $\sim \geq 20\mu\text{m}$)), according to their cross-sectional area. In rat these

classifications were: small, $<900\mu\text{m}^2$ (diameter $\sim <30\mu\text{m}$); and large, $\geq 900\mu\text{m}^2$ (diameter $\sim \geq 30\mu\text{m}$) (S. D. Dib-Hajj et al., 1998).

2.4 RESULTS

2.4.1 VALIDATION OF COLONIC FAST BLUE LABELLING OF DRG NEURONES

The specificity of Fast Blue uptake by thoracolumbar colon-projecting afferents was first investigated qualitatively. Following transcardial perfusion with fixative (4% PFA), dorsal root ganglia from mice and rats were removed from spinal levels thoracic 3 (T3) – T13, lumbar 1 (L1)-L4. The distal colon was also removed at this time. The colon was opened along the antimesenteric border and multiple light and fluorescent images were taken and combined together to determine the position of Fast Blue (FB) injection sites relative to likely splanchnic and pelvic afferent receptive fields. In Figure 6A, multiple FB injection sites can be seen as a faint yellow under light microscopy (red arrows). During surgery, efforts were made to ensure injection sites were made primarily within regions of the colon likely to be innervated by the lumbar splanchnic nerve (LSN), therefore FB injections were not made more distally. Once microdissected, individual DRG from FB-labelled mice and rats were imaged under fluorescent microscopy to confirm uptake of FB into cells (Figure 6B). In DRG T5-L2, fluorescing white cells could be seen under UV illumination, with maximal frequency in L1 (Figure 6B&C). Importantly, none, or very few (<2 cells/DRG) retrograde-labelled cells were observed in DRG T4, L3 and L4 (Figure 6B&C). This is consistent with the expression pattern observed in previous studies and provides confirmation that off-target labelling of non-visceral tissues is unlikely to have occurred (Christianson et al., 2006; Robinson et al., 2004). As such, no alterations to surgical procedures (e.g. position or

number of injection sites, concentration or amount of FB injected) were made other than to optimise survival time post-surgery (see below).

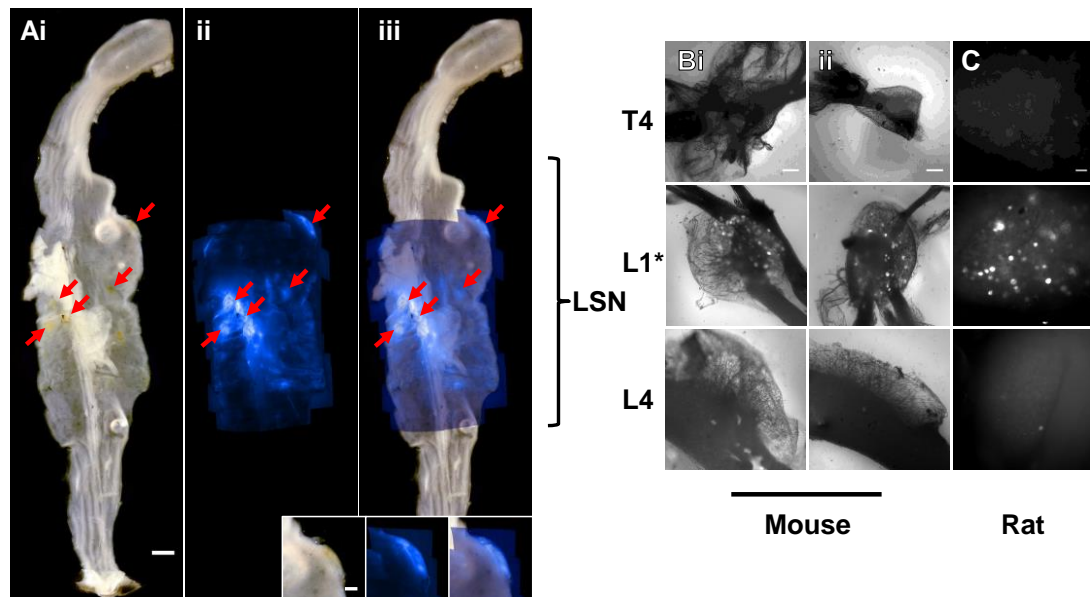


Figure 6. Validation of colonic Fast Blue labelling of DRG neurones. Fast Blue (FB) injection into the colon wall of mouse and rat and retrograde transport to dorsal root ganglia. A Mouse colon from anus (bottom) to splenic flexure (top) was removed post-fixation (4% PFA) and opened along the anti-mesenteric border. Multiple light (i) and fluorescent (ii) images were taken and combined to form this representative image. Example FB injection sites have been highlighted (red arrows). For illustrative purposes, typical afferent innervation of the lumbar splanchnic (LSN) of the colon is indicated. Scale bar 3mm. *Inset*, close-up of FB injection site. Scale bar 1mm. Representative light and fluorescent images of whole DRG removed from retrograde-labelled mice (Bi & ii) and rat (C). DRG from spinal level L1 contains significant fluorescing FB-labelled cells, corresponding to the splanchnic afferent pathway, whilst DRG T4 and L4 contain none, or very few (<2 cells/DRG), fluorescing cells. *L2 for rat (C) is shown due to species differences in maximal LSN innervation. Scale bars 100 μ m.

2.4.2 QUANTITATIVE ASSESSMENT OF FB COLONIC LABELLING OF DRG NEURONES

To further optimise the retrograde-labelling procedure, two survival time points post-surgery were investigated: 3 and 7 days. A shorter post-surgery period was chosen to minimise the duration of post-operative care and potential stressors exposed to the animal (e.g. twice daily analgesic injections). A shorter survival had also been shown previously to produce comparable labelling frequencies, and to increase neuronal intensity of FB (Perry & Lawson, 1998). DRG from mice, having undergone surgery 3 or 7 days prior, were removed and counts were made of neuronal FB-positive (FB+) profiles from DRG sections. At the 7 day time point, FB+ cells made up $8.1 \pm 0.0\%$ of the total profiles counted across all DRG ($N = 2$)(Figure 7). In DRG with maximal frequency of colonic projecting neurones, this increased to $13.3 \pm 0.0\%$ for L1 (Figure 7). Numbers of FB+ cells in 3-day survival time animals were comparable when examining counts from T13 and L1 DRG ($N = 3-4$, Figure 7), although due to the low N number for 7-day survival time mice statistical analysis was not conducted. Size-frequency analysis of neuronal profiles from T13 & L1 DRG (3-day survival time) revealed a broad distribution of FB+ cells, with a larger area mean ($619 \pm 62 \mu\text{m}^2$) compared to all T13 & L1 profiles ($442 \pm 18 \mu\text{m}^2$, $N = 4$, Figure 7). The distribution revealed the majority of FB+ profiles as small or medium in size (cross sectional area $<800 \mu\text{m}^2$) with very few cells considered large (cross sectional area $>800 \mu\text{m}^2$).

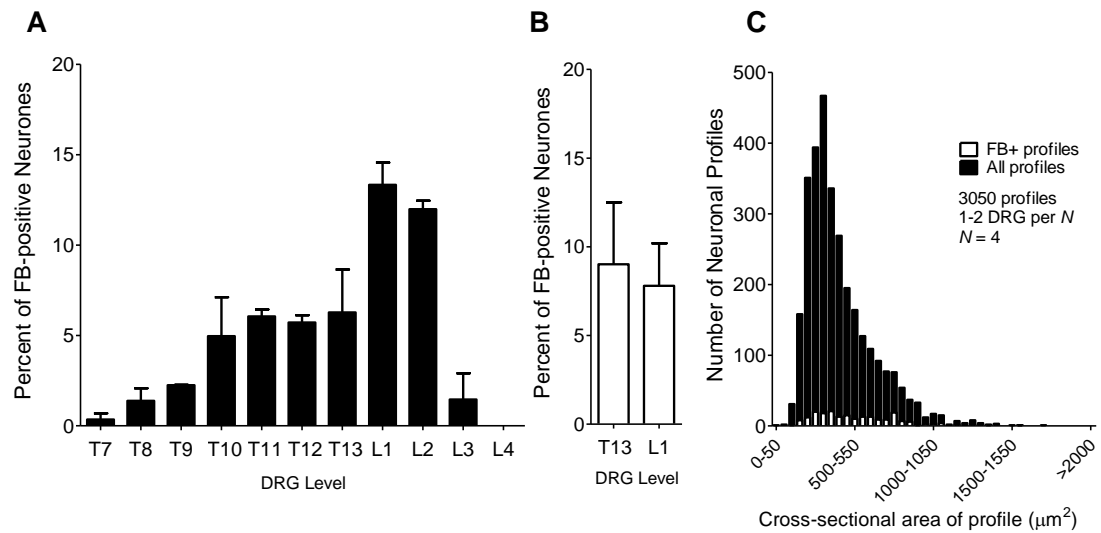


Figure 7. Effect of survival time on FB colonic labelling of mouse DRG neurones. A Distribution of FB labelling across DRG levels from T7-L4 in mouse with peak frequency at L1, reflecting the lumbar splanchnic afferent innervation, with 7 days survival time. B Distribution of FB labelling across DRG levels T13 and L1 in mouse after 3 days survival time. C Cross-sectional area-frequency histogram of FB+ profiles superimposed on all T13&L1 DRG neuronal profiles from 3 day survival mice.

2.4.3 RETROGRADE FAST BLUE LABELLING OF COLONIC TISSUES IS UNAFFECTED BY *Nav1.9* KNOCK-OUT

In later studies, *Nav1.9* ^{-/-} mice were used to investigate the functional role of *Nav1.9* in visceral afferent pathways and to confirm the specificity of *in situ* hybridisation techniques. Therefore in order to confirm that *Nav1.9* knock-out was not altering the extent of colonic innervation by extrinsic afferent fibres, comparisons of the proportions of FB+ profiles from *Nav1.9* ^{+/+} and *Nav1.9* ^{-/-} mice were made. During comparable surgical procedures and survival times (3 days), the extent of FB labelling of T13 & L1 DRG did not significantly differ (unpaired t-test, $p = 0.48$, Figure 8B). Indeed, size-frequency analysis revealed similar distributions (2-way ANOVA, $p = 0.11$) and area means (*Nav1.9* ^{+/+}: $619 \pm 62 \mu\text{m}^2$ vs *Nav1.9* ^{-/-}: $628 \pm 33 \mu\text{m}^2$, unpaired t-test, $p = 0.90$) of FB+ profiles from *Nav1.9* ^{+/+} and *Nav1.9* ^{-/-} mice.

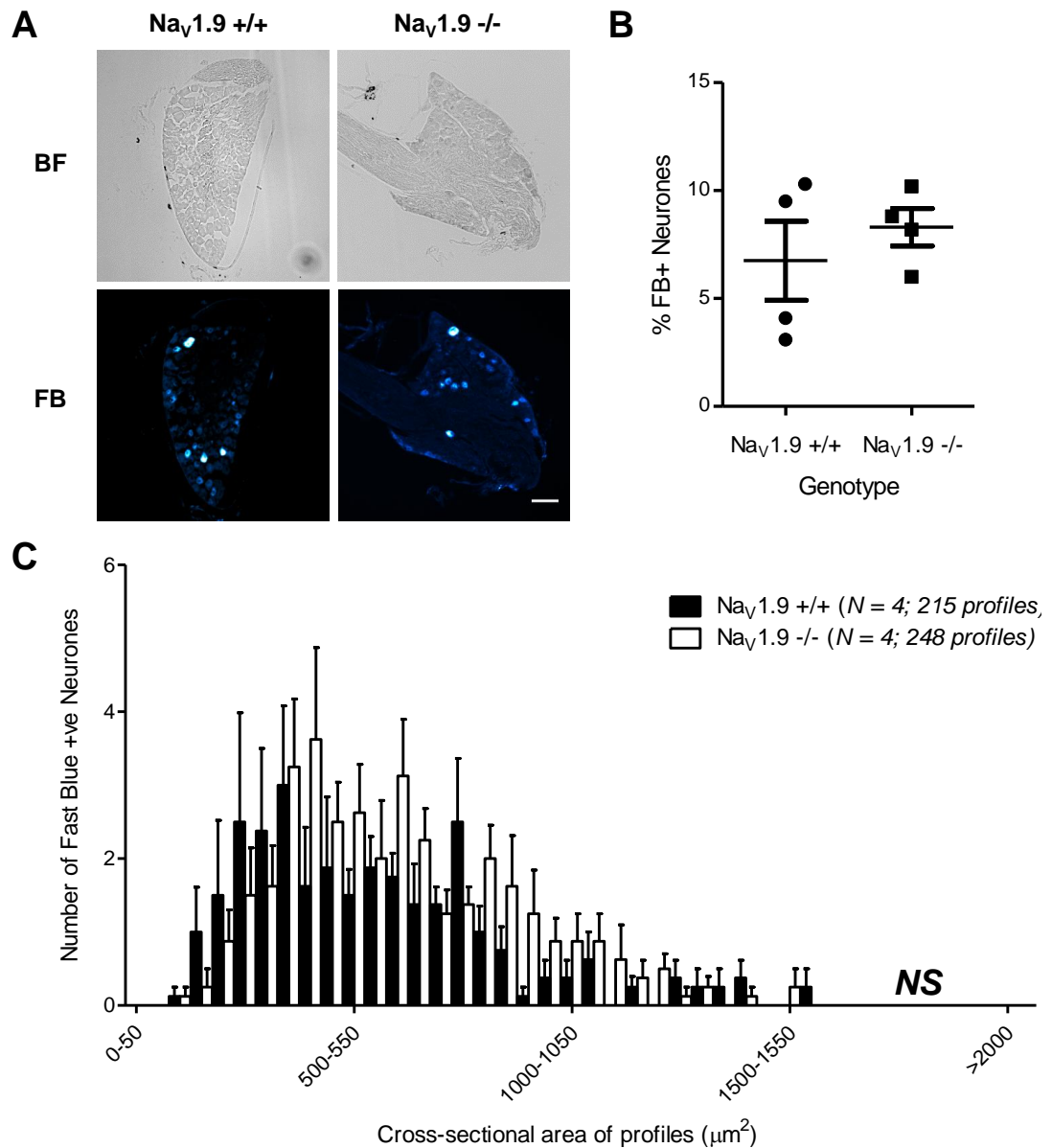


Figure 8. Comparison of FB colon labelling from Na_v1.9 +/+ and Na_v1.9 -/- mice. Comparison of total number of colon-projecting FB labelled neuronal profiles in Na_v1.9 +/+ and Na_v1.9 -/- mice from DRG T13 and L1. A Example brightfield and fluorescent images of Na_v1.9 +/+ and Na_v1.9 -/- DRG sections showing FB labelling of cells. Scale bar 100μm. B Scatter plot of FB+ profiles as a percentage of total profiles (mean of 1-2 DRG per animal, N = 4 per genotype). C Mean frequency of cross-sectional area of profiles (in 50μm² divisions) from Na_v1.9 +/+ and Na_v1.9 -/- mice.

2.4.4 EXPRESSION OF *Nav1.9* IN MOUSE COLON-PROJECTING DRG NEURONES BY *IN SITU* HYBRIDISATION

Next the expression of *Nav1.9* mRNA transcripts in colon-projecting thoracolumbar DRG sections of mouse was examined using *in situ* hybridisation. The thoracolumbar spinal innervation of the colon was studied because this is thought to transduce predominantly nociceptive stimuli from the colon. Under polarised light, *Nav1.9*-positive staining was observed as clusters of silver grains visible over cells and was present in $69.0 \pm 3.0\%$ of all neurones (Figure 9). Injections of FB retrograde tracer into the colon wall labelled $6.7 \pm 1.8\%$ of neurones in *Nav1.9* $+/+$ mice (Figure 9A) and of these, $50.5 \pm 3.3\%$ expressed *Nav1.9* transcripts. Size-frequency analysis of all neurones revealed that *Nav1.9* mRNA was expressed to a higher extent in small ($<400 \mu\text{m}^2$ cross-sectional area, $83.4 \pm 2.9\%$) compared to larger ($\geq 400 \mu\text{m}^2$, $50.2 \pm 2.9\%$) neurones (Figure 9E). The cross-sectional area mean of *Nav1.9*-positive (*Nav1.9*+) neurones was also significantly smaller than that of all profiles (*Nav1.9*+ profiles, $378 \pm 12 \mu\text{m}^2$ vs all profiles, $442 \pm 18 \mu\text{m}^2$, unpaired t-test, $p < 0.05$, $N = 4$). In the FB+ population, a similar, although less robust trend towards expression of *Nav1.9* in small ($71.1 \pm 10.9\%$) rather than larger cells ($44.5 \pm 3.6\%$) was observed (Figure 9D). Area mean of *Nav1.9*+/FB+ profiles did not significantly differ from those of FB+ profiles (*Nav1.9*+/FB+ profiles, $553 \pm 56 \mu\text{m}^2$ vs FB+ profiles, $619 \pm 62 \mu\text{m}^2$, unpaired t-test, $p = 0.46$, $N = 4$). Interestingly, the relative intensity of *Nav1.9* mRNA staining was significantly greater in the FB- compared to FB+ neuronal population (unpaired t-test, $p < 0.0001$, Figure 9F).

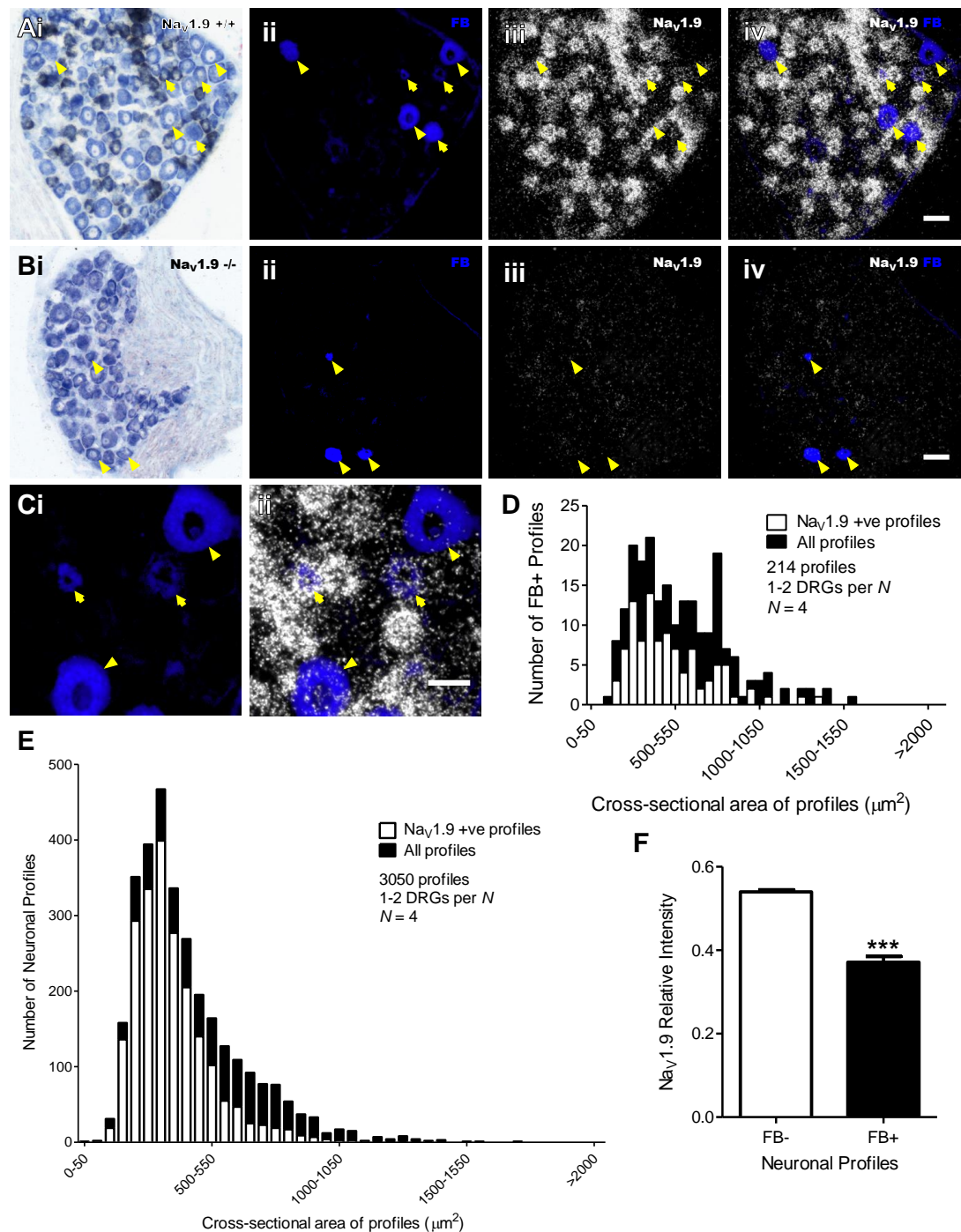


Figure 9. Expression of $Na_v1.9$ mRNA in colon-projecting DRG neurones in mouse. Radiographic *in situ* hybridisation of $Na_v1.9$ mRNA transcript expression (bright-field with Giemsa counterstain, i; polarised light, iii) in thoracolumbar dorsal root ganglia section from $Na_v1.9^{+/+}$ (A) and $Na_v1.9^{-/-}$ (B) mice combined with retrograde labelling of sensory neurones from the colon with Fast Blue (FB only, ii; merge, iv; scale bar 50 μm). C Expanded top-right region of $Na_v1.9^{+/+}$ ganglia section (A) showing four retrogradely labelled sensory neurones (Scale bar 30 μm). Yellow arrows indicate colon projecting neurones positive for $Na_v1.9$ mRNA expression. Yellow arrowheads indicate colon projecting neurones negative for

Na_v1.9 mRNA expression. Cross-sectional area histogram of FB+ (D) and all (E) neuronal profiles from Na_v1.9 +/+ mice showing Na_v1.9 mRNA transcript expression super-imposed on top (white bars). F Relative intensity of Na_v1.9 mRNA transcript expression in FB-negative (FB-) versus FB-positive (FB+) neuronal profiles (3050 profiles, $N = 4$, unpaired t-test, *** $p < 0.0001$).

The specificity of *in situ* hybridisation for Nav1.9 was confirmed by identical expression distribution patterns for Nav1.9 mRNA using three separate Nav1.9 probes (Probe 1, 64.7% Nav1.9+; Probe 2, 61.8% Nav1.9+; Probe 3, 73.5% Nav1.9+, Figure 10A). Probe 3 labelled less efficiently than both Probe 1 and Probe 2, and was not used in subsequent studies. Furthermore, controls using an excess of unlabelled probe on Nav1.9 +/+ sections produced only background signal (Figure 10B). And finally, expression was not observed in Nav1.9 -/- sections (Figure 9B).

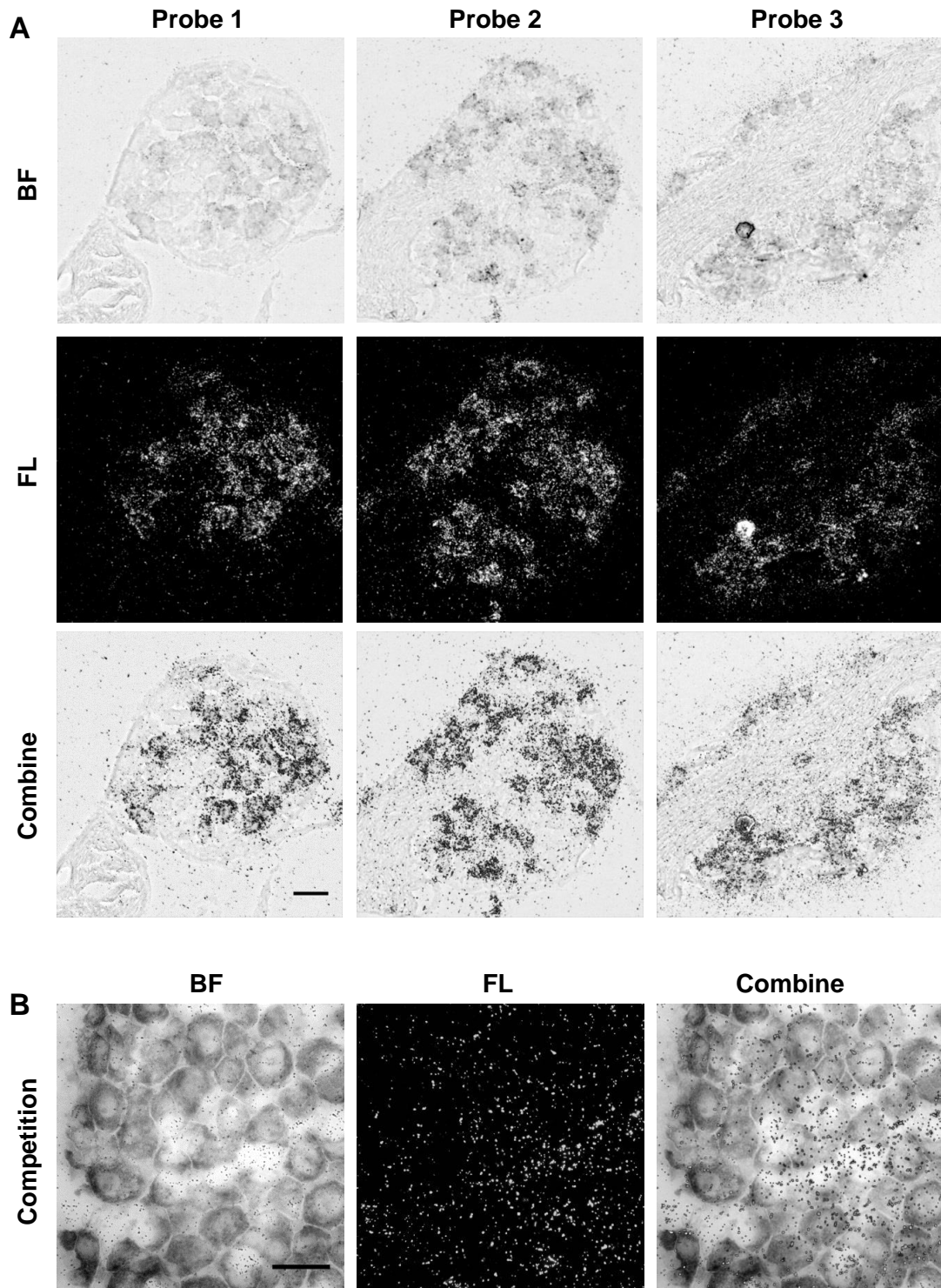


Figure 10. *In situ* hybridisation controls. A Similar expression patterns were detected using three different $\text{Na}_v1.9$ mRNA probes on $\text{Na}_v1.9$ +/+ DRG sections. Fluorescence (FL) polarised-light images of silver-grains were superimposed on brightfield (BF) images. Scale bar 100 μm . B Control *in situ* hybridisation reactions with excess unlabelled probe produced only background signal. Scale bar 50 μm .

2.4.5 EXPRESSION OF Nav1.9 IN RAT COLON-PROJECTING DRG NEURONES BY IMMUNOHISTOCHEMISTRY

To further characterise Nav1.9 expression and confirm mRNA distribution, a commercially available Nav1.9 antibody was used in rat DRG sections, in conjunction with two protein markers of neuronal subpopulations: CGRP and IB4. Both markers, CGRP, present in small peptidergic nociceptors and IB4, expressed in small nonpeptidergic nociceptors, have been used previously to characterise viscerally projecting neuronal populations (Robinson & Gebhart, 2008). Multiple commercially available polyclonal Nav1.9 antibodies (Alomone Labs [ASC-017], Sigma Aldrich [S2196] and abcam [ab84086]) were initially characterised on both mouse and rat DRG tissue. Of these, Alomone Labs Nav1.9 antibody performed most effectively, producing expression patterns predicted from existing literature (Amaya et al., 2000). The use of the Alomone Lab antibody in rat DRG tissue had been previously validated by both the manufacturer and in the literature (Y. Q. Yu, Zhao, Guan, & Chen, 2011); however the specificity of the selected antibody in mouse tissues was unknown. As such, pilot labelling experiments were performed on mouse DRG sections of both Nav1.9 +/+ and Nav1.9 -/- animals. Non-specific labelling was observed in Nav1.9 -/- tissue suggesting that the Alomone Lab antibody was not useful in specifically labelling Nav1.9 in mouse tissues. Therefore subsequent experiments were performed in rat DRG tissue. Nav1.9 immunoreactivity was observed in a similar proportion of total neurons to ISH experiments ($64.5 \pm 2.4\%$, Figure 11A), with a significantly smaller area mean compared to all profiles ($642 \pm 14 \mu\text{m}^2$ vs $767 \pm 25 \mu\text{m}^2$, unpaired t-test, $p < 0.05$). No specific staining was seen in control experiments following preincubation with excess

antisera peptide for Nav1.9 antibody or when primary Nav1.9 antibody was omitted (Figure 11). Furthermore, western blot analysis of Nav1.9 immunoreactivity on rat DRG lysates identified a principal band at the predicted molecular weight for Nav1.9 of ~205kDa; two fainter bands at larger molecular weights could also be observed, possibly reflecting glycosylated forms of the channel (Figure 11). Colonic injection of FB labelled $9.2 \pm 1.6\%$ of rat sensory neurons, and in agreement with ISH data, of this FB+ population, $51.9 \pm 5.8\%$ of cells stained positive for Nav1.9 (Figure 12). The area mean of FB+ profiles was almost equal to that of all cells ($786 \pm 86 \mu\text{m}^2$ vs $767 \pm 25 \mu\text{m}^2$, unpaired t-test, $p = 0.84$). Further, in congruence with ISH studies, the relative intensity of Nav1.9 immunoreactivity was significantly less in FB+ versus FB-neuronal profiles (unpaired t-test, $p < 0.0001$).

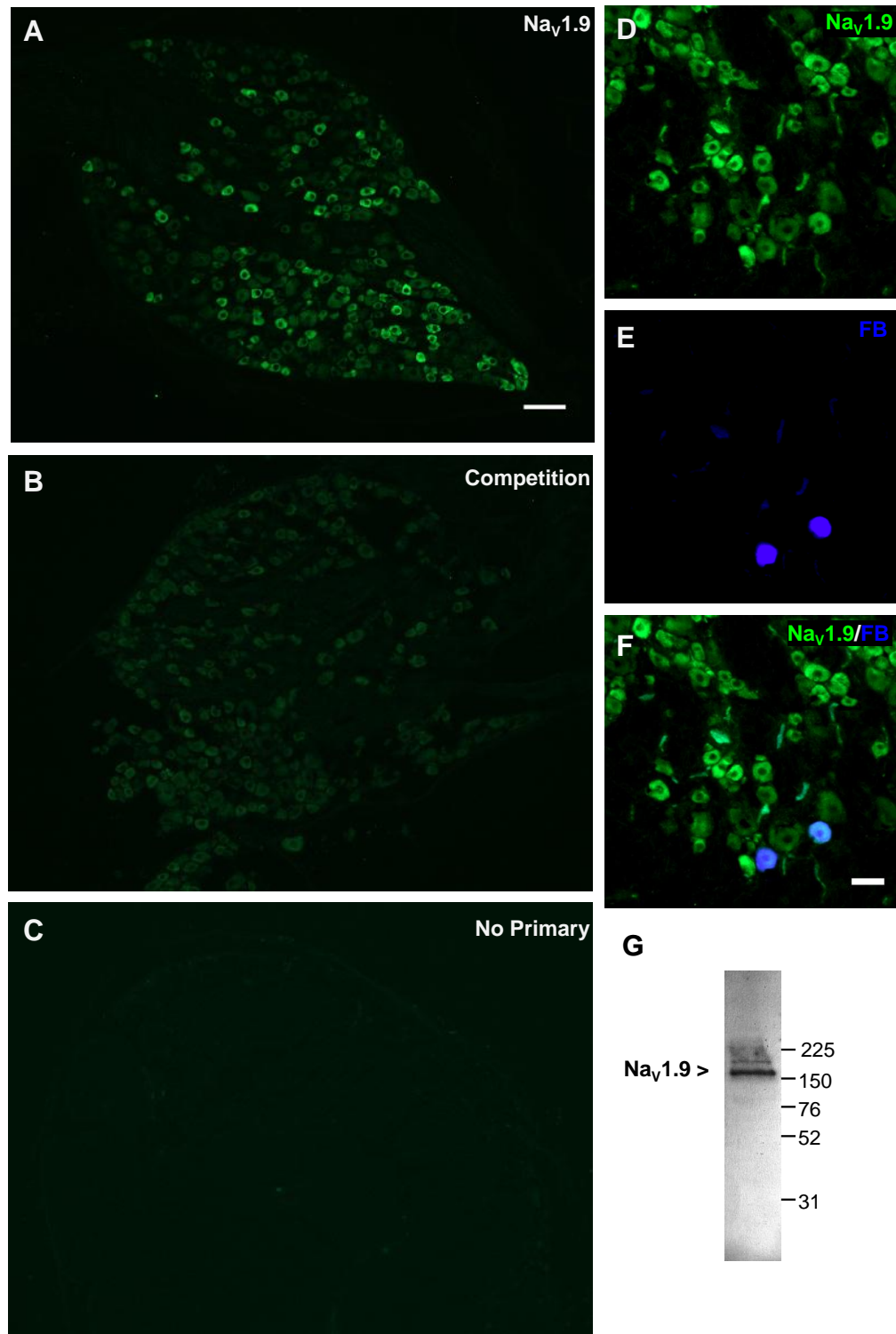


Figure 11. Example Na_v1.9 protein expression in rat colon-projecting DRG neurones. A Na_v1.9 immunoreactivity (IR) on DRG section. Pre-incubation controls with excess protein antisera (B) and without primary Na_v1.9 antibody (C) resulted in only background signal. Scale bar 100 μm. High magnification images of Na_v1.9-IR (D), FB tracer (E) and merge (F). Scale bar 50 μm. G Western blot analysis of Na_v1.9-IR on rat DRG lysates showing a band at the predicted molecular weight of ~205 kDa.

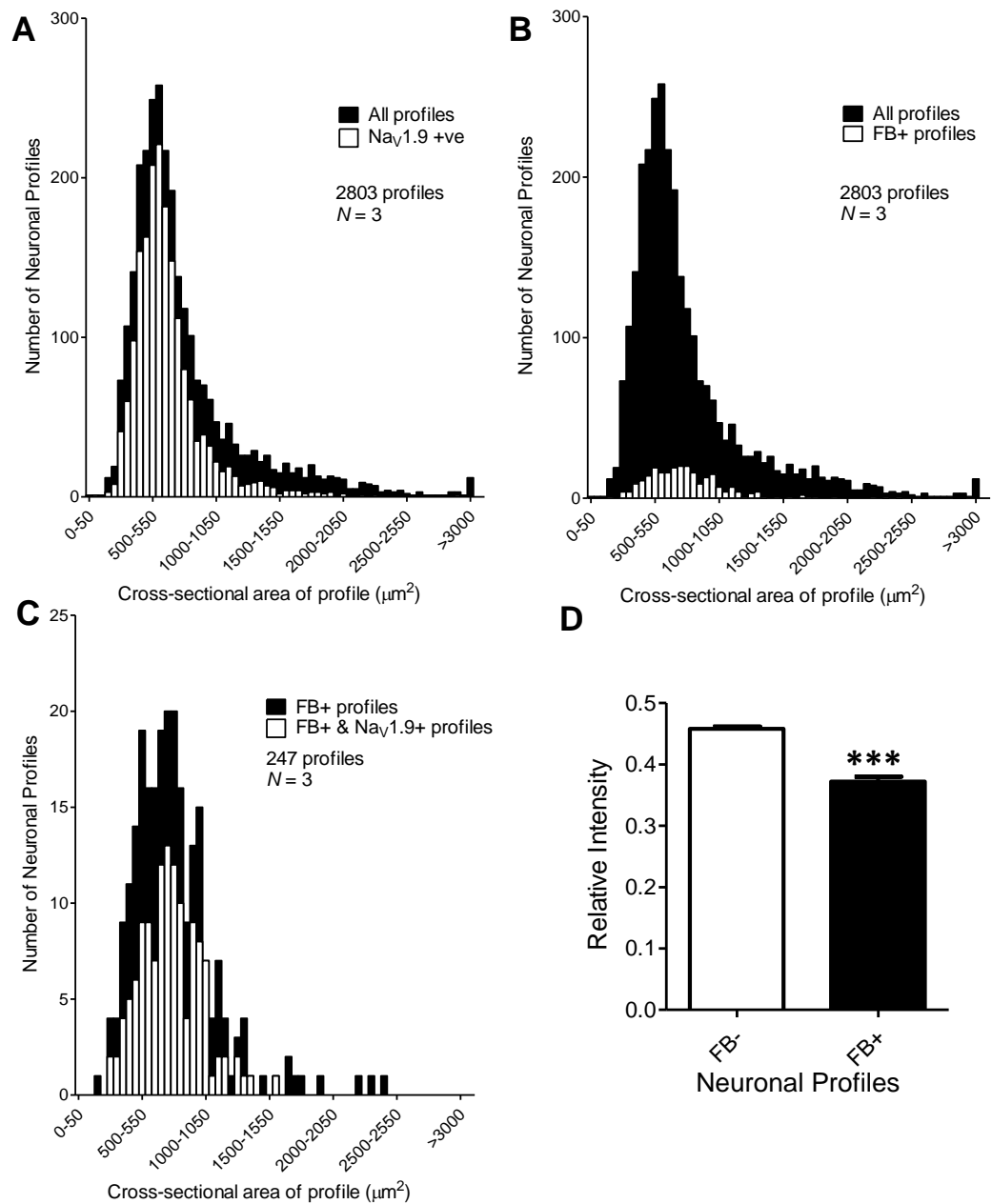


Figure 12. Quantitation of $\text{Na}_V1.9$ protein expression in rat colon-projecting DRG neurones. Cross-sectional area histogram of $\text{Na}_V1.9$ -positive ($\text{Na}_V1.9$ +; A) and FB+ (B) neuronal profiles superimposed on all profiles. C Cross-sectional area histogram of FB+/ $\text{Na}_V1.9$ + profiles superimposed on FB+ profiles. D Comparison of relative intensities of $\text{Na}_V1.9$ + profiles from FB- and FB+ populations (2803 profiles, $N = 3$, $***p < 0.0001$).

2.4.6 CO-EXPRESSION OF $Na_v1.9$ WITH CGRP AND IB4 IN RAT COLON-PROJECTING DRG NEURONES BY IMMUNOHISTOCHEMISTRY

The protein markers, CGRP and IB4 were used to further characterise neuronal populations. IB4 binding (IB4+) occurred in $51.4 \pm 1.6\%$ of all cells, predominantly in small neurones (96.6% of area $< 900\mu m^2$ neurones are IB4+), such that the area mean of IB+ profiles is significantly smaller than that of all cells ($559 \pm 17 \mu m^2$ vs all profiles, unpaired t-test, $p < 0.01$, Figure 14Ai). In FB+ neurones, IB4 binding was observed in a smaller population compared to all neurones ($26.0 \pm 5.3 \%$, Table 5), consistent with the enriched peptidergic population present in colon-projecting neurones. This is also revealed by a greater proportion of CGRP-expressing cells in the FB+ population (FB+/CGRP+: $80.6 \pm 8.3\%$) compared to all neurones ($34.8 \pm 1.1\%$, Figure 14Bii). The expression of CGRP is localised to comparatively smaller neurones than the average neuronal area ($639 \pm 26 \mu m^2$ vs all profiles, unpaired t-test, $p < 0.05$, Figure 14Bi).

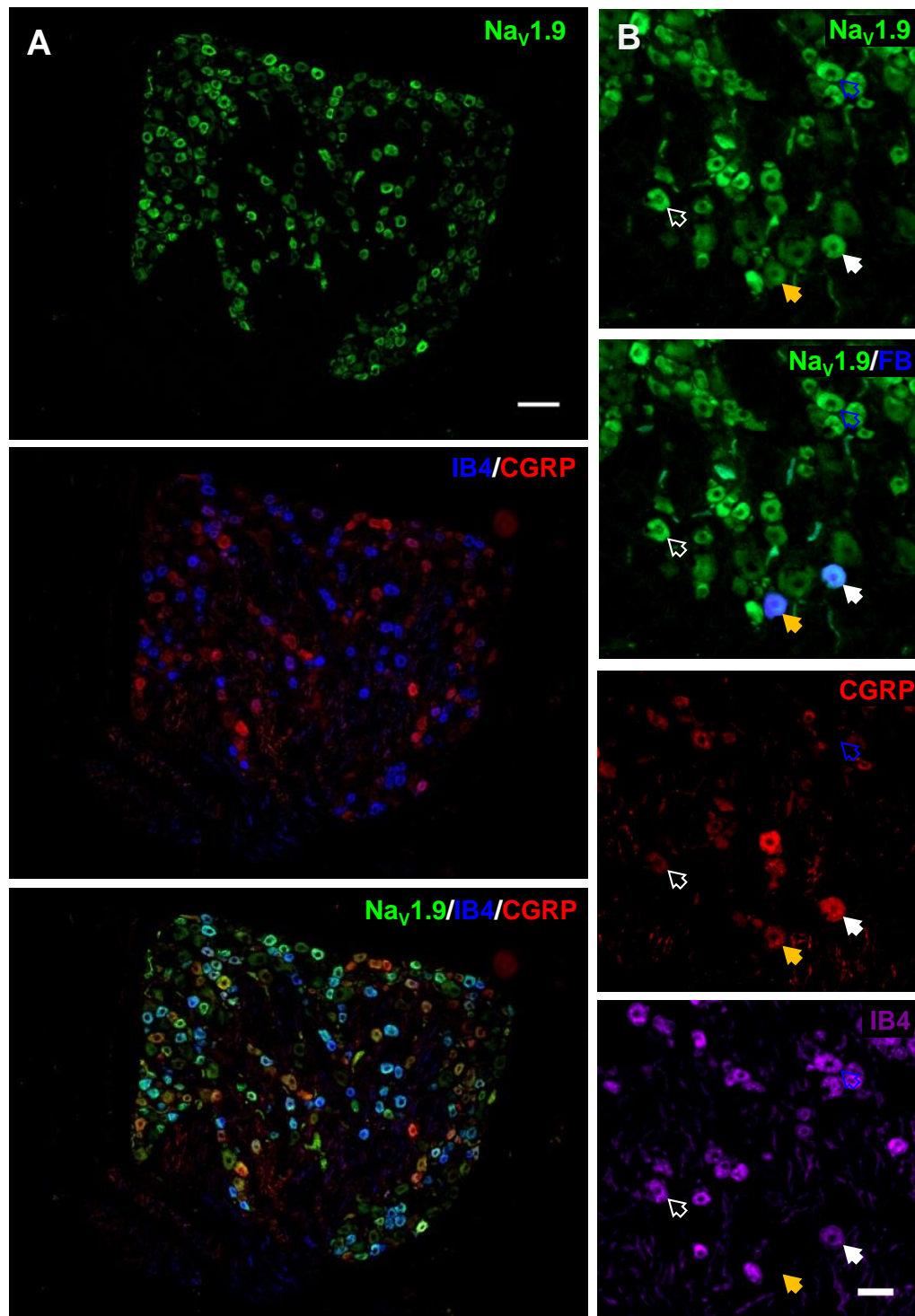


Figure 13. Co-expression of Na_v1.9 with neuronal protein markers, IB4 and CGRP. A Multiple fluorescent immunohistochemistry for Na_v1.9, CGRP and IB4 in dorsal root ganglia from rat. Scale bar 100µm. B High magnification IHC for Na_v1.9, CGRP and IB4 combined with retrograde labelling of sensory neurons from the colon. Filled arrows indicate FB+ve neurons colocalising for Na_v1.9, CGRP and IB4 (white) or Na_v1.9 and CGRP (orange). Open arrows indicate example FB-ve neurons colocalising for Na_v1.9, CGRP and IB4 (white) or Na_v1.9 and IB4 (blue). Scale bar 50µm.

<i>In situ hybridisation</i>						
	Colonic (FB+; <i>N</i> = 4, <i>n</i> = 214)			Unlabelled (FB-; <i>N</i> = 4, <i>n</i> = 2836)		
Nav1.9 expression	50.5 ± 3.3 %			69.0 ± 3.0 %		
<i>Immunohistochemistry</i>						
	Colonic (FB+; <i>N</i> = 4, <i>n</i> = 247)			Unlabelled (FB-; <i>N</i> = 4, <i>n</i> = 2556)		
Nav1.9-IR	51.9 ± 5.8 %			65.9 ± 3.0 %		
CGRP-IR	80.6 ± 8.3 %			30.0 ± 0.4 %		
IB4-binding	26.0 ± 5.3 %			54.1 ± 0.9 %		
	% of Nav1.9	% of CGRP		% of Nav1.9	% of CGRP	
Nav1.9 & CGRP-IR	89.6 ± 7.8%	57.7 ± 5.1 %		32.7 ± 1.1%	71.7 ± 3.5 %	
	% of Nav1.9	% of IB4		% of Nav1.9	% of IB4	
Nav1.9-IR & IB4 binding	34.7 ± 7.3%	68.5 ± 7.7 %		73.4 ± 3.5%	89.2 ± 2.1 %	
	% of Nav1.9	% of CGRP	% of IB4	% of Nav1.9	% of CGRP	% of IB4
Nav1.9 & CGRP-IR & IB4 binding	32.9 ± 7.4%	21.5 ± 4.9%	64.2 ± 4.0%	16.9 ± 2.6%	36.5 ± 4.4%	20.2 ± 2.3%
	% of CGRP	% of IB4		% of CGRP	% of IB4	
CGRP-IR & IB4 binding	31.1 ± 5.4 %	95.7 ± 4.4 %		42.8 ± 5.5 %	23.7 ± 2.9 %	

Table 5. Summary of Nav1.9 expression and colocalisation with IB4 and CGRP protein markers. *In situ* hybridisation was performed isotopically in mouse. Immunohistochemistry was performed in rat.

The colocalisation of CGRP and IB4 on unlabelled (FB-) neurones is relatively infrequent and two distinct populations can be identified by these markers (see Table 5: 23.7 ± 2.9 % of FB-/IB4+ profiles are also CGRP+, Figure 15A).

Interestingly, in FB+ neurones, the vast majority of IB4+ neurones also express CGRP (95.7 ± 4.4 % of FB+/IB4+ profiles are also CGRP+), and those that do not express CGRP are Nav1.9+. As such, it is not possible to split colon-projecting neurones into two distinct populations based on CGRP and IB4 expression.

Rather, colon-projecting neurones should be considered a non-homogenous population of CGRP-positive neurones; a quarter of which also co-express IB4.

Similar to Nav1.9 expression levels, the relative intensity of IB4-binding was significantly reduced in FB-/IB4+ neurones compared to FB+/IB4+ neurones (unpaired t-test, $p < 0.0001$, Figure 14Aiii). Interestingly, the relative intensity of CGRP-immunoreactivity did not differ between FB- and FB+ neuronal populations (unpaired t-test, $p = 0.084$).

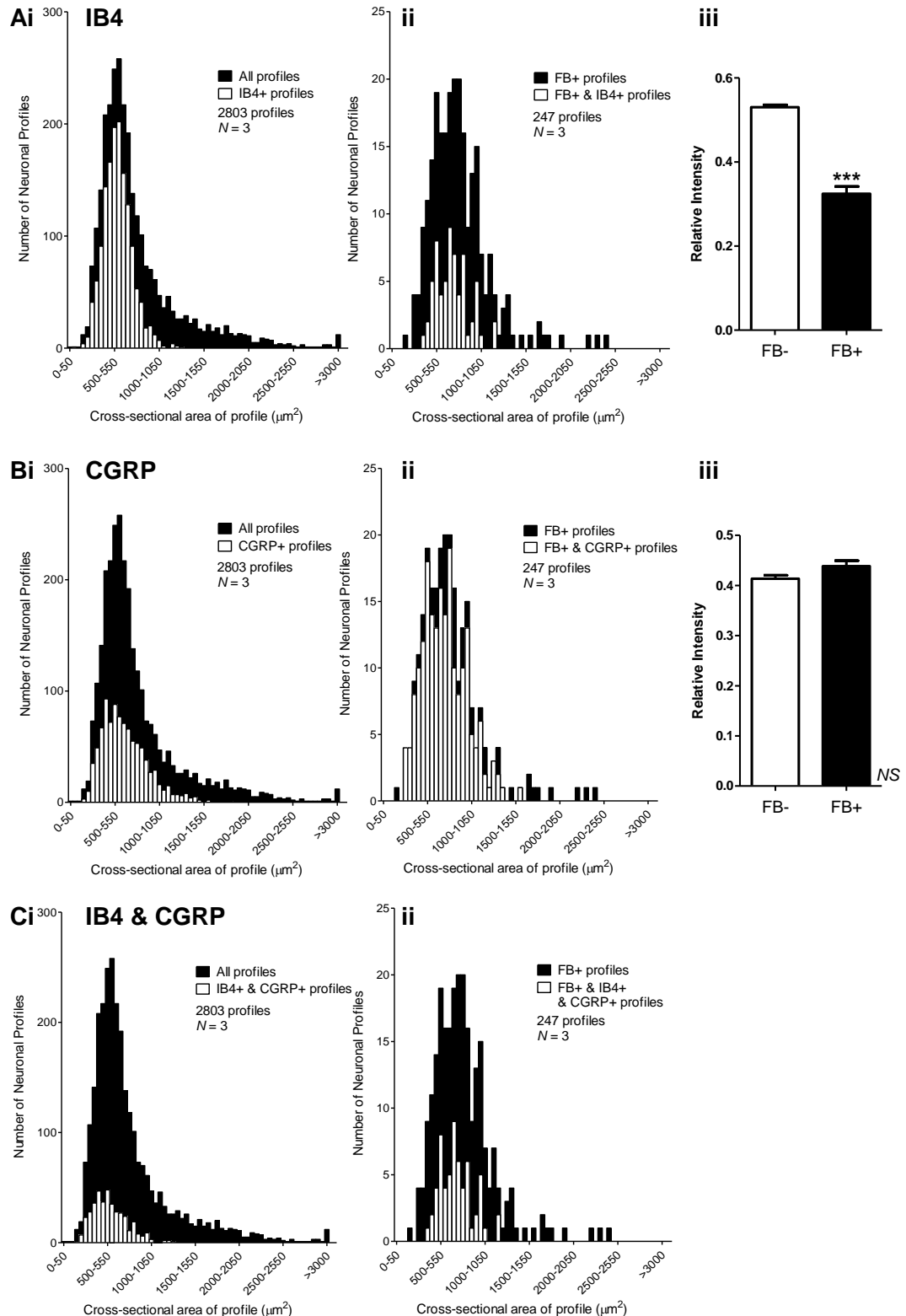


Figure 14. Histograms showing cross-sectional area of IB4+, CGRP+ and IB4&CGRP+ neuronal profiles. Cross-sectional area histogram of IB4+ (Ai), CGRP+ (Bi) and CGRP&IB4+ (Ci) superimposed on all neuronal profiles. FB+ specific labelling by IB4, CGRP and colocalisation of both is shown in (ii) of A, B and C. Comparison of relative intensities of IB4+ (Aiii) and CGRP+ (Biii) profiles from FB- and FB+ populations. *** $p < 0.0001$

The extensive colocalisation of Nav1.9 with IB4 staining predicted by previous studies was also observed in FB- neurons ($73.4 \pm 3.5\%$ of Nav1.9+ profiles; Table 5) (Fang et al., 2006). By contrast, Nav1.9/IB4 colocalisation was less extensive ($34.7 \pm 7.3\%$ of Nav1.9+ profiles) in FB+ colonic populations, with an enrichment of colocalisation with CGRP (from FB-: $32.7 \pm 1.1\%$ to FB+: $89.6 \pm 7.8\%$ of Nav1.9+ profiles, Figure 15). It is worth bearing in mind that the unlabelled (FB-) population of neurones will also contain a small proportion of colon-projecting neurones that have not been successfully labelled by the Fast Blue tracer injections. The differences in Nav1.9 expression relative to CGRP and IB4 protein markers in colon-projecting (FB+) and unlabelled (FB-) neurones is clearly exemplified in the proportional Venn diagrams shown in Figure 15.

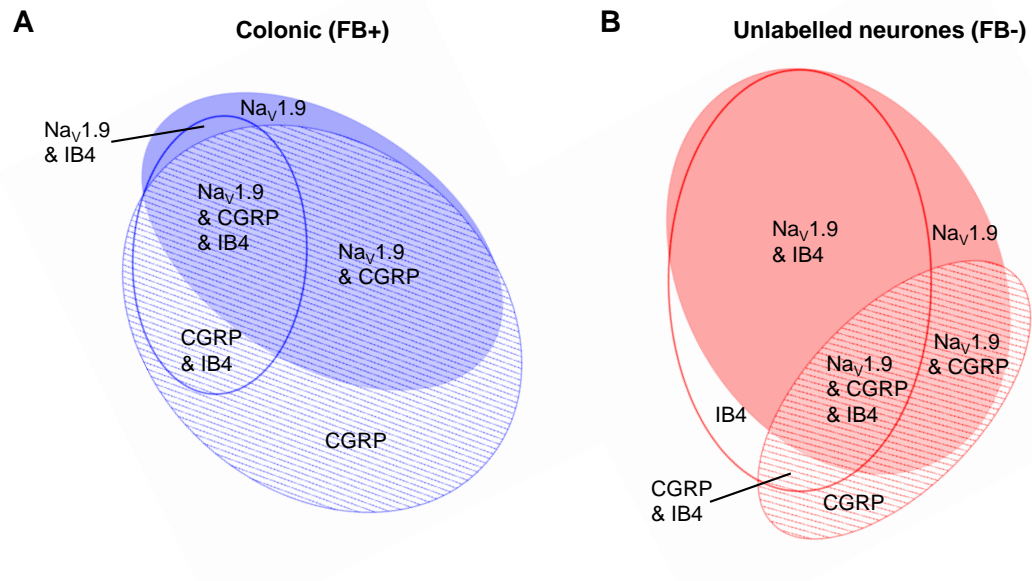


Figure 15. Colocalisation of Na_v1.9, CGRP and IB4 in neuronal populations. Proportional Venn diagram of colocation of Na_v1.9, CGRP and IB4 in colonic (FB+; A) and unlabelled (FB-; B) neuronal populations in rat. Approximately 13.3% of FB+ and 21.6% of FB- neurones were negative for Na_v1.9, CGRP and IB4.

2.4.7 NON-ISOTOPIC IN SITU HYBRIDISATION TO DETERMINE EXPRESSION OF *Nav1.9* mRNA TRANSCRIPTS

A secondary *in situ* hybridisation method was also used to verify isotopic techniques. Using a RNA oligomer signal amplification system and an enzymatic chromogenic reaction, greater sensitivity could be achieved with DRG sections. Importantly, using this technique yielded comparable results to those from isotopic techniques, but in a greatly attenuated experimental timeframe (8 hours vs. 3-4 weeks for isotopic ISH). As such, in a single *Nav1.9* *+/+* DRG section, 65.6% of profiles expressed *Nav1.9* mRNA transcripts, with 66.7% of small cells positive for *Nav1.9* (Figure 16). Interestingly, the degree of probe binding and subsequent chromogenic reaction product was heavily influenced by protease pre-treatment of DRG sections, with greater signals observed following 30 minute pre-treatments (Figure 16). No signal was seen without any protease pre-treatment (Figure 16). In isotopic ISH techniques, adequate signal was observed without protease pre-treatment, although signal-to-noise ratio may have been improved by short protease digestion, to further reveal sense complementary bases.

In *Nav1.9* *-/-* sections, chromogenic signal was also dependent on protease digestion. Whilst no specific signal above background had been observed in isotopic ISH experiments in *Nav1.9* *-/-* sections (Figure 9), protease pre-treatment and chromogenic ISH did reveal mRNA expression (Figure 16). The intracellular localisation of mRNA signal, however, differed greatly from that seen in *Nav1.9* *+/+* sections (Figure 16). Staining was observed predominantly in the nucleus of cells, with very limited expression seen in cytoplasmic regions (consisting of punctate isolated brown DAB particles), compared to a more

homogeneous cytoplasmic and nuclear expression distribution in Nav1.9 +/+ sections. This is consistent with reduced nuclear export and processing of mutant pre-mRNA, which may be occurring given the loss of exons 4 and 5 from the *SCN11A* allele in Nav1.9 -/- mice, and the subsequent production of mutant mRNA (see Chapter 3, 3.3.1 *Nav1.9 knock-out mice* for more details). It is interesting to note that isotopic techniques were unable to detect the nuclear signal above background, suggesting either an increased dependence on protease digestion or increased sensitivity of the chromogenic amplification techniques.

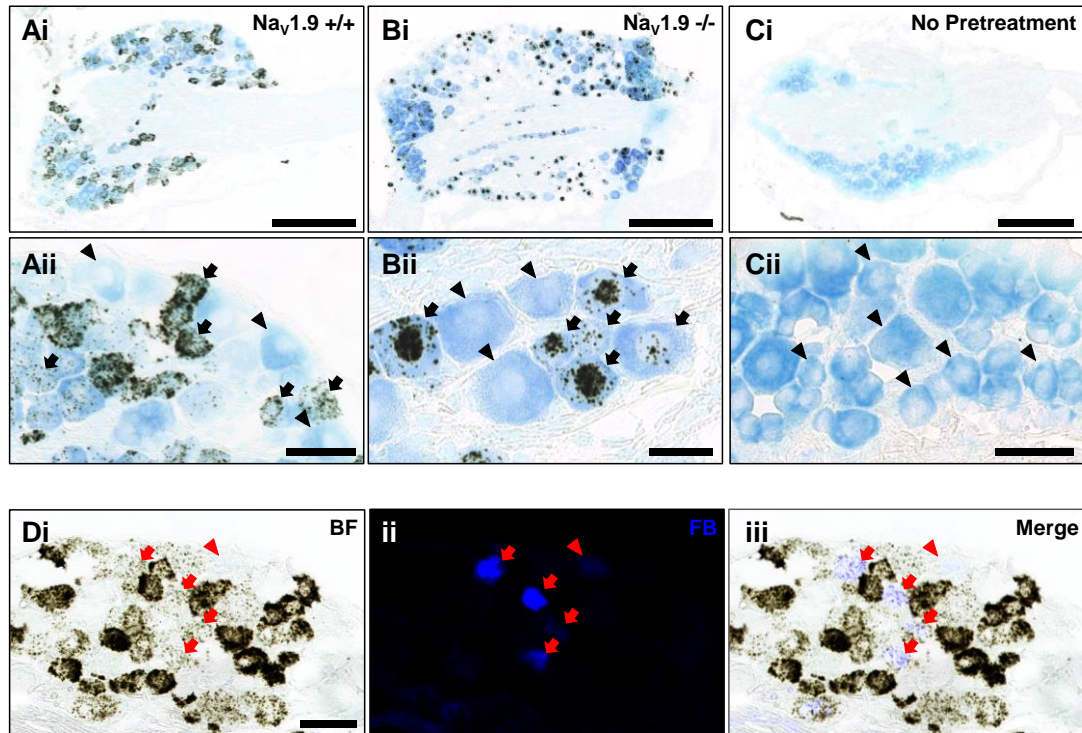


Figure 16. Non-isotopic *in situ* hybridisation of Nav1.9 in mouse DRG sections. Low magnification images of DAB chromogenic reaction products (brown) of Nav1.9 mRNA *in situ* hybridisation on protease pre-treated (20 mins) Nav1.9 +/+ (Ai) and Nav1.9 -/- (Bi) mouse DRG sections counterstained with Giemsa. Scale bar 250µm. High magnification images of same sections (Nav1.9 +/+, Aii (scale bar 50µm) and Nav1.9 -/-, Bii (scale bar 25µm)). No protease pre-treatment is shown in C (i, low mag (scale bar 250µm); ii, high mag (scale bar 50µm)) on Nav1.9 +/+ DRG sections. Examples of positively labelled cells are highlighted with arrows, whilst unlabelled cells are marked with arrowheads. D Chromogenic ISH of Nav1.9 +/+ section (i, protease pre-treatment 30 mins) with colon-labelled Fast Blue neuronal profiles (ii) and merge (iii). Scale bar 50µm. Examples of FB+ neurones with positive labelling for Nav1.9 are highlighted with red arrows and FB+ neurones negative for Nav1.9 labelling are highlighted with red arrowheads.

2.5 SUMMARY OF KEY FINDINGS

2.5.1 *VALIDATION AND QUALIFICATION*

1. Injection of retrograde neuronal marker Fast Blue into the colon of mouse and rat labelled a population of neurones within thoracolumbar DRGs
2. There was no significant difference in degree of labelling in harvesting DRG after 3 days compared to 7 days from Fast Blue injection
3. Fast Blue injection into the colon labelled approximately 5-10% of DRG neurones, the vast majority of which were small-medium in size.
4. No difference was observed in the extent or size-frequency distribution of Fast Blue DRG neurone labelling from Nav1.9 +/+ and Nav1.9 -/- mice

2.5.2 *IN SITU HYBRIDISATION FOR Nav1.9 mRNA TRANSCRIPT EXPRESSION*

1. Nav1.9 mRNA was observed in $69.0 \pm 3.0\%$ of all mouse DRG neurones. Over 80% of small neurones expressed Nav1.9 mRNA.
2. Of Fast Blue-positive colon-projecting neurones, Nav1.9 mRNA was expressed in $50.5 \pm 3.3\%$ of DRG neurones.
3. Using isotopic in situ hybridisation, DRG sections from Nav1.9 -/- mice did not express Nav1.9 mRNA signal above background levels.
4. The Nav1.9 was expressed by a range of FB+ neuronal cell sizes. Those FB+ profiles expressing Nav1.9 had a comparable area mean to all FB+ profiles.
5. The degree of expression of Nav1.9 mRNA was significantly less in colon-projecting neurones compared to non-colon-projecting neurones.

2.5.3 EXPRESSION OF $Na_v1.9$ PROTEIN BY IMMUNOHISTOCHEMISTRY AND CO-EXPRESSION WITH PROTEIN MARKERS, CGRP AND IB4

1. 65.9 ± 3.0 % of profiles exhibited $Na_v1.9$ -immunoreactivity in rat DRG sections, the majority of which could be considered small in size.
2. In congruence to ISH experiments, $Na_v1.9$ protein was expressed by just over half of colon-projecting DRG neurones in rat. The FB+ neuronal population was predominantly small-medium in size and had an area mean comparable to that of all profiles.
3. Extensive colocalisation of $Na_v1.9$ with IB4 staining was also observed in FB- neurons, with three-quarters of $Na_v1.9$ + neurones also expressing IB4. By contrast, $Na_v1.9$ /IB4 colocalisation was less widespread ($\sim 30\%$) in FB+ colonic populations.
4. IB4 binding was less frequent in FB+ neurones ($\sim 25\%$) compared to FB- neurones ($\sim 50\%$). Although when it was expressed by a FB+ neurones, it was almost always ($>95\%$) co-expressed with CGRP.
5. The vast majority of colon-projecting neurones expressed CGRP compared to only about a third of all neurones, indicative of the presence of an enriched peptidergic population.
6. Interestingly, whilst the relative intensity of $Na_v1.9$ and IB4 was on average significantly less in FB+ neurones, the relative intensity of CGRP+ neurones was comparable to FB- neurones.

2.6 DISCUSSION

The data presented here demonstrates significant expression of Nav1.9 within a subset of colon-projecting visceral afferent fibres. Firstly, the retrograde labelling of colon-projecting afferent fibres by Fast Blue injection into the serosal wall of the distal colon was validated.

2.6.1 VALIDATION OF RETROGRADE LABELLING OF COLON IN MOUSE AND RAT

Colon labelled neurones were identified in DRG from mouse and rat. Labelled neurones were observed in DRG from thoracolumbar regions of the spinal cord (T7-L3) with a modal peak at L1 (*n.b.* L2 for rat). Importantly, DRG L4 and L5, which receive mainly somatosensory input from the leg, were void of retrograde-labelled cells. This is in line with previous studies examining colon projecting sensory neurones (Christianson et al., 2006; Robinson et al., 2004). Kyloh *et al.* provide an association between the position of dye injection sites along the colon and the DRG primarily retrogradely labelled in mouse (Kyloh et al., 2011). In the distal colon (9-15mm from anus) of mouse, labelling was seen predominantly in DRG of the lumbosacral regions of the spinal cord (L6-S1), whilst in mid to proximal colon dye injections (30-75mm from anus), labelling was primarily of the thoracolumbar levels (T6-L2) (Kyloh et al., 2011). The labelling of T7-L3 populations observed in the current study is in line with the range of injection sites over the mid to mid-distal colon (~17–50mm from anus; see Figure 6). This population of visceral afferents represents a distinct anatomical pathway innervating the colon, with sensory afferent fibres originating in thoracolumbar DRG tracking with the lumbar splanchnic nerve

(Brierley et al., 2004; Jänig & Morrison, 1986). This pathway has unique mechanosensory characteristics that transduce differing physiological sensations. For example, thoracolumbar visceral afferents encode pain and discomfort, alongside spinal intestino-intestinal reflexes. This differs from more distal lumbosacral visceral afferents, which act to regulate the evacuation and storage functions of the pelvic organs, in conjunction with transducing physiological and pathophysiological sensations (Jänig, 1996). This is further reflected in the differences in mechanosensory fibre types present in thoracolumbar and lumbosacral visceral populations (Brookes et al., 2013; Song et al., 2009; Zagorodnyuk et al., 2010). The cross-sectional area of dye-labelled colon-projecting neurones was consistent with previous studies and represented a predominantly small to medium neuronal size, consistent with C – A δ fibre types (Beyak et al., 2004; Christianson et al., 2006; Robinson et al., 2004). Reduction in retrograde labelling survival times from 7 days post-surgery to 3 days post-surgery provided adequate labelling within the primary region of interest: thoracolumbar DRG. By reducing survival times it was possible to minimise handling of the animal and optimise the experimental design. Significant retrograde labelling from visceral tissues had been previously shown at 2-3 days (Perry & Lawson, 1998).

2.6.2 EXPRESSION OF $Na_v1.9$ IN COLON-PROJECTING VISCERAL AFFERENT FIBRES

Having confirmed that surgical microinjection of Fast Blue into the colon wall of mice and rats was labelling visceral afferent fibres consistent with the known anatomical innervation of the lumbar splanchnic nerve; identical procedures

were performed on Nav1.9 $-/-$ mice. Whilst no overt phenotype, other than diminished hyperalgesia to inflammatory stimuli, had been described for Nav1.9 $-/-$ mice, retrograde labelling confirmed that no loss or lesion of visceral sensory neurones had occurred (Ostman et al., 2008; Priest et al., 2005). The relative numbers of FB+ neurones and their cross-sectional area distributions were comparable between homozygous genotypes. This suggests that subsequent functional effects observed in Nav1.9 $-/-$ mice were due to activity of the channel rather than changes in the developmental innervation of mouse tissues. Whilst developmental changes resulting from knock-out of Nav1.9 cannot be discounted, in addition to comparable colonic innervation, Nav1.9 $-/-$ mice also exhibit normo-mechanosensation in behavioural models, suggesting this is unlikely to be the case (Martinez & Melgar, 2008; Priest et al., 2005; Ritter et al., 2009). Furthermore, this shows that Nav1.9 $-/-$ DRG sections could be used as a negative control for expression analysis.

In all sensory neurones from thoracolumbar DRG, Nav1.9 expression was observed in approximately 69% by both *in situ* hybridisation and immunohistochemistry. The specificity of both techniques was confirmed by multiple controls, including staining in sections from Nav1.9 $-/-$ mice and the use of competition controls. Furthermore, experiments where *in situ* hybridisation was combined with immunohistochemistry indicated a correlation between expression of Nav1.9 mRNA and Nav1.9 protein immunoreactivity, although this was not studied in detail (see Appendix 1). Importantly, the extent of Nav1.9 expression observed in the current study was very similar to previous studies (Table 3) and suggests that the techniques

utilised were reliably labelling Nav1.9. Any disparity between the current study (ISH: 69% Nav1.9+) and those undertaken previously (ISH: 58-69% Nav1.9+) may be attributable to species differences (e.g. Dib-Hajj *et al.* and Fukuoka *et al.* both performed ISH in rat), the anatomical regions under investigation (e.g. nearly all studies have been performed on L4 DRG) and/or methodological considerations relating to the design of the ISH probe, the IHC antibody used, and the determination of thresholds for positive staining (S. D. Dib-Hajj *et al.*, 1998; Fang *et al.*, 2002; Fukuoka *et al.*, 2008). Efforts were taken to ensure that a non-arbitrary objective threshold was used in both ISH and IHC experiments (see 2.3.7). Nav1.9 expression by ISH was also shown in the vast majority of small neurones (~80%) in line with previous studies (see Table 3 (S. D. Dib-Hajj *et al.*, 1998; Fukuoka *et al.*, 2008)). It is important to note that Nav1.9 expression is not restricted to small diameter cells and approximately 25% of medium and large cells also express Nav1.9.

In colon-projecting sensory neurones, approximately 50% expressed Nav1.9 by *in situ* hybridisation or immunohistochemistry. The mean cross-sectional area of FB+/Nav1.9+ neurones did not significantly differ from that of FB+ cells, suggesting a relatively even distribution within the population (see Figure 9 and Figure 11). The extent of Nav1.9 labelling, as determined by the development of silver grains during *in situ* hybridisation and immunoreactive products during immunohistochemistry techniques, was significantly less (~15%) in colon-projecting cells compared to non-colon-projecting cells. It suggests, at least in a naïve state, that whilst Nav1.9 is expressed in FB+ neurones, proportionally it is not expressed to the same extent as in other cells.

This lower somal expression of Nav1.9 may, to some extent, explain the challenges observed in recording Nav1.9 currents in isolated colon-projecting neurones (Beyak et al., 2004; Gold et al., 2002). However, patch-clamp methodologies are likely to represent the greatest influence on observations of Nav1.9 current *in vitro* (Cummins et al., 1999; Rugiero et al., 2003).

Isotopic *in situ* hybridisations performed on Nav1.9 -/- DRG sections did not show any signal over background. This was confirmed in DRG sections from multiple mice and using Probes 1 and 2 independently, as well as in a separate experiment using a combination of both Probes 1 and 2 (Figure 10). In contrast, subsequent experiments using non-isotopic chromogenic *in situ* hybridisations to label Nav1.9 -/- sections did see significant expression of Nav1.9 mRNA. To explain this disparity, it is necessary to examine how the Nav1.9 -/- mice utilised in this study were developed. In the Nav1.9 knock-out originally developed by Ostman *et al.*, bases 538-764, relating to exons 4 & 5 of the Nav1.9 mRNA, were replaced with a neomycin selection cassette (Ostman et al., 2008). This led to a frameshift mutation in the mRNA and the introduction of a stop codon during translation, and as such the Nav1.9 channel is not expressed. Importantly, the truncated Nav1.9 mRNA is still transcribed by sensory neurones as seen by RT-PCR and the sequencing of reaction products (Ostman et al., 2008). As the isotopic probes used in the present study are complementary to regions of Nav1.9 mRNA unaffected by genetic manipulation (Probe 1: bases 968-1001 & Probe 2: 2641-2674), it is likely that these would still bind accessible Nav1.9 mRNA in Nav1.9 -/- DRG sections. However, the lack of expression in Nav1.9 -/- DRG sections suggests that Nav1.9 mRNA is not able

to be accessed by these isotopic probes. This is in agreement with the observed intracellular expression pattern of Nav1.9 mRNA in Nav1.9 -/- DRG sections following non-isotopic chromogenic *in situ* hybridisations. Here, chromogen deposits are predominantly located within the nucleus, with very low levels observed in the cytoplasm. This is consistent with the incomplete processing of the transcribed Nav1.9 pre-mRNA into a nuclear export-ready mature mRNA (Alberts, Wilson, & Hunt, 2008). This nuclear expression is only revealed by protease treatment and the partial digestion of fixed cellular proteins. As such, it may be that isotopic *in situ* hybridisations were unable to access the nuclear pre-mRNA sufficiently to provide a signal in Nav1.9 -/- DRG sections. The non-isotopic chromogenic technique may be more sensitive due to the protease treatment and can therefore label these oligomers. Furthermore, due to the proprietary nature of the non-isotopic chromogenic *in situ* hybridisation methodology (Advanced Cell Diagnostics RNAScope 2.0), the exact complementary sequences of the probes used are unknown and likely contribute to the differences in specificities observed between techniques. Further studies using protease pre-treatments prior to isotopic *in situ* hybridisations may reveal a similar nuclear retention in Nav1.9 -/- sections. Furthermore, the co-labelling for nuclear export factors may help to rationalise why nuclear export efficiency is reduced.

2.6.3 CO-EXPRESSION OF NAV1.9 WITH PROTEIN MARKERS CGRP AND IB4

The protein markers, CGRP and IB4, have been used extensively in the past to identify small diameter peptidergic and non-peptidergic nociceptive populations, respectively. The characterisation of CGRP-positive or IB4-positive

visceral afferents as nociceptors has not been comprehensively studied. However, the vast majority of visceral afferents are likely to transduce stimuli into the noxious range and, as such, these markers will be relevant to visceral nociceptors. In congruence with existing literature, over 80% of FB+ neurones in the current study expressed CGRP and 25% bound IB4, representative of the predominant peptidergic innervation of the colon (Christianson et al., 2006; Perry & Lawson, 1998; Robinson et al., 2004; Song et al., 2009). Importantly, unlike somatic-projecting C-fibres, IB4 did not label a separate population to CGRP in colon-projecting neurones. In fact, almost all FB+/IB4+ neurones were also positive for CGRP (see Figure 15 and Table 5). Original studies highlighting the differences between peptidergic and non-peptidergic primary afferent pathways suggest very limited co-expression of protein markers for these two populations (*n.b.* previously fluoride-resistant acid phosphatase (FRAP), now IB4) (Hunt & Rossi, 1985; Nagy & Hunt, 1982). However, it is clear from more recent studies that CGRP and IB4 are not mutually exclusive and some overlap in expression does occur (Ambalavanar & Morris, 1992; Bergman et al., 1999; Hwang, Oh, & Valtschanoff, 2005). Both Robinson *et al.* and Christianson *et al.* report similar colocalisation of CGRP and IB4 in colon-projecting neurones (Christianson et al., 2006; Robinson et al., 2004). Indeed, Hwang *et al.* suggests that CGRP+ and IB4+ neurones represent largely the same population in bladder-projecting neurones, markers that were previously thought to be independent (D. L. Bennett, Dmietrieva, Priestley, Clary, & McMahon, 1996; Hwang et al., 2005); this is supported by data generated from the current study.

These classifications are important as nociceptor sub-populations differ anatomically and rely on different growth factors during development and maturity (Snider & McMahon, 1998). However, whilst somatic peptidergic and non-peptidergic primary afferents project centrally to restricted termination sites within the spinal cord (lamina I and lamina II, respectively) and likely play differing roles in nociception, visceral primary afferents exhibit far more divergent central projections across lamina I, V and X (and occasionally contralaterally to lamina V) and have far less functional specialisation (Hunt & Rossi, 1985; Jänig, 1996; Sugiura et al., 1989). The high, and convergent, expression of nerve growth factor (NGF) receptor TrkA within CGRP+ visceral neurones highlights their reliance on this trophic factor (D. L. Bennett et al., 1996; McMahon, Armanini, Ling, & Phillips, 1994). This is particularly relevant to sodium channel expression, where Nav1.9 expression has been shown to be regulated by glia cell line-derived neurotrophic factor (GDNF), rather than NGF, in sensory neurones (Cummins, Black, Dib-Hajj, & Waxman, 2000; Fjell et al., 1999). Although Trk receptor subtypes are predominantly expressed in viscerally projecting afferent fibres, there is evidence for GDNF receptor expression within a population of high-threshold TRPV1-positive visceral afferents (D. L. Bennett et al., 1996; Malin et al., 2009; McMahon et al., 1994). Whilst the extent of Nav1.9 expression is reported to correlate with IB4 binding, a significant subset of TrkA-positive neurones are also Nav1.9-positive (Fang, Djouhri, et al., 2005; Fang et al., 2006). As stated in the introduction, Nav1.9 is expressed by both peptidergic and nonpeptidergic non-visceral neuronal populations of C-fibres. The present data shows that in colon-projecting neurones, approximately 90% of Nav1.9+ cells express CGRP and a third of

these also bind IB4. A speculative extrapolation of these data would suggest that colon projecting neurones primarily consist of populations dependent on NGF and a combination of NGF and GDNF; and Nav1.9 is expressed by both of these populations. However, a more comprehensive study is required to understand this patterning, using specific, not surrogate, markers for growth factor dependence and the inclusion of other known growth factors, e.g. BDNF and TrkB.

Given the established correlation between IB4 binding and GDNF receptor expression, it is tempting to infer that Nav1.9 is also expressed by the subset of TRPV1+ high threshold units identified by Malin *et al.* (Malin et al., 2009). Interestingly, a knock-out of GFR α 3 in mouse ameliorates inflammation-induced colorectal afferent hypersensitivity; a phenotype that would fit with our current understanding of Nav1.9 function (Amaya et al., 2006; Tanaka, Shinoda, Feng, Albers, & Gebhart, 2011). However, single-unit intracellular recordings of colon sensory neurones using dye-fills and post-functional labelling for Nav1.9 will be required to confirm this association.

The current study quantifies the extent of Nav1.9 expression within the lumbar splanchnic afferent pathway and provides colocalisation data with key markers of nociceptive populations. That Nav1.9 is expressed by approximately half of colon-projecting neurones indicates functional specialisation. The role of Nav1.9 in specific fibre types is studied in greater detail in subsequent chapters.

CHAPTER 3: THE ROLE OF $\text{Na}_v1.9$ IN MECHANOSENSITIVITY OF GUT VISCERAL AFFERENT FIBRES

3.1 INTRODUCTION

3.1.1 SENSATION EVOKED BY MECHANICAL ACTIVATION OF VISCERAL AFFERENTS

The mechanosensitivity of visceral afferents is central to the relay of sensation in response to bowel distension, stretch or mesenteric traction. Specifically, pain is thought to arise following distension or contraction around a faecal bolus in the partially or fully occluded bowel (Brookes et al., 2013). The sensitivity of visceral afferents to mechanical stimuli can be significantly modulated by inflammatory mediators. Indeed, visceral hypersensitivity to barostat balloon distension is observed in a subset of irritable bowel syndrome (IBS) patients (Mertz, Naliboff, Munakata, Niazi, & Mayer, 1995). In fact, the diagnosis of functional gastrointestinal disorders using the ROME III criteria relies extensively on visceral pain as an important criterion (Foundation, 2006). In pain arising from the GI tract, the activation of visceral primary afferents is relayed to the brain by multiple pathways, including the spinoreticular, spinomesencephalic, spinohypothalamic and spinothalamic tracts (Almeida, Roizenblatt, & Tufik, 2004). Conscious sensation is mainly mediated via the spinothalamic tract, with thalamic projections to the insula, anterior cingulate cortex and somatosensory cortex (SI/II) (Almeida et al., 2004). Collectively, these pathways provide localisation and intensity of the stimulus, alongside affective pain behaviours and integration into higher autonomic motor responses. The spinoreticular, spinomesencephalic and spinohypothalamic tracts mainly activate unconscious or autonomic responses to visceral sensory input. These pain pathways can be robustly activated by balloon distension of

the gastrointestinal tract. In man, distension to pressures of 15 to 40mmHg in the upper colon (>16cm above the anal sphincter) and small intestine evokes graded pain, which is abolished following bilateral sympathectomy from T7 to L3 (Bentley & Smithwick, 1940; Ray & Neill, 1947). By contrast, pain caused by distension of the anal canal (within 16cm of the sphincter) is unaffected by sympathectomy, highlighting the dual innervation of the GI tract (splanchnic versus pelvic nerves) (Ray & Neill, 1947). In an attempt to model visceral pain, many surrogates of consciously perceived pain have been used in animals. These include pseudoaffective and visceromotor reflexes *in vivo*, visceral afferent nerve firing *ex vivo* and changes in phosphorylation states of transcription factors or intracellular signalling proteins relevant to nociceptive processing (e.g. ERK). In addition, various types of stimuli have been used to activate visceral pain pathways, such as slow ramp versus rapid phasic distension by balloon inflation or fluid fill of the bowel, chemically-induced contraction of the bowel around a fixed inflated balloon and *ex vivo* blunt probe, circumferential stretch and stroke of discrete receptive fields of afferent fibres. Together these methodologies have enabled the successful identification of numerous biochemical and mechanistic pathways relevant to human visceral pain. However, it is important to understand both the limitations of and the rationale behind the use of each stimulus in order to maximise its utility when interrogating visceral afferent function; in this case specifically the role of Nav1.9. Functionally, how Nav1.9 deletion impacts afferent fibres of the splanchnic nerve has been investigated and is reported below. The afferent subtypes present in the splanchnic nerve and the types of stimuli used to stimulate them are discussed in more detail.

3.1.2 VISCERAL AFFERENT SUBTYPES

The terminals of visceral afferents transduce mechanosensory function, such as contraction, relaxation and distortion of the bowel wall, movement of the mesentery, evacuation of the rectum and movement of gas. They are also responsible for the transduction of pain, as well as the regulation of local blood flow and reflexes. These were described in detail in Chapter 1. Here, the major afferent subtypes present in the splanchnic nerve are reiterated; of which there are four characterised functionally: serosal, mesenteric, muscular and mucosal.

3.1.2.1 Mucosal afferents

Mucosal afferents are activated by light stroking or compression of the mucosa but not distension or contraction of the gut (Blackshaw & Gebhart, 2002; Brierley et al., 2004). The arborisation of endings in the mucosal villi and in close proximity to the epithelial lining of the GI tract enable mucosal afferents to encode physiological information relating to luminal content as well as osmotic and pH changes (Grundy, 2002). Spinal mucosal afferent terminals are found in both thoracolumbar splanchnic and lumbosacral pelvic pathways, although are significantly more frequent in the latter (Brierley et al., 2004). They appear to serve a similar nutrient and luminal content-sensing function as vagal mucosal afferent more proximally located.

3.1.2.2 Muscular afferents

Evidence from flat-sheet colonic preparations indicate very few (< 10%) afferent fibres responsive to low intensity stretch in the splanchnic nerve (Brierley et al., 2004). This is in contrast to significant proportions of

lumbosacral afferent endings identified in the internal anal sphincter and rectum that are sensitive to low distension pressures (<20mmHg (Malin et al., 2009)) and low intensity stretch stimuli (<3g (Hughes et al., 2009)). Interestingly, during *in vivo* recordings of jejunal mesenteric afferents from vagotomised animals approximately 50% of single units responded to low distension pressures and continued to encode into the noxious range; characteristics used in other studies to define muscular afferents (Booth et al., 2008). This suggests that a significant proportion of thoracolumbar spinal afferents may be responsive to low distension pressures and highlights the methodological differences attributed to the use of stretch in a flat-sheet preparation versus distension in a tubular preparation. The structure and location of thoracolumbar muscular afferent endings remain to be determined.

3.1.2.3 Spinal serosal and mesenteric afferents

Spinal serosal and mesenteric afferents represent the vast majority (80-90%) of splanchnic afferent fibres identified by functional studies (Brierley et al., 2004; Lynn & Blackshaw, 1999). These endings are strongly associated with branch points of mesenteric arteries and can be activated by local compression of both the mesentery and the wall of the gut (Brierley et al., 2004; Song et al., 2009). They act to encode traction on the mesentery and contraction and distension of the gut wall (Bessou & Perl, 1966; Jänig, 1996). These endings are sensitive to ischaemia, hypoxia and capsaicin and represent a major type of nociceptor (Haupt et al., 1983; Longhurst & Dittman, 1987; Longhurst et al., 1984). Serosal and mesenteric afferents respond to much higher intensities of colorectal distension (>40mmHg (Brierley et al., 2008)) and stretch (>9g (Hughes et al.,

2009)), in addition to compression of their receptive field by von Frey hair probe. Recent evidence of 'serosal' afferents associated with vascular vessels penetrating as far as the submucosa in the colon wall of guinea-pig suggests that this nomenclature may be misleading. As of yet detailed structure-function anatomical studies have not been performed in mouse (Hughes et al., 2009; Song et al., 2009). It should be noted that following dye fills of pelvic nerve rectal IGLE-like structures were only clearly distinguishable in guinea-pig but not mouse. This suggests that anatomical differences exist between these species, which may also extend to other spinal afferent sub-classes including serosal (Olsson et al., 2004; Spencer, Kerrin, Singer, et al., 2008).

As well as being associated with arterial branching points of the mesentery, these endings continue into the colon wall and innervate arteries and second-order arterioles of the submucosa (Song et al., 2009). As well as providing mechano-transduction sites intra- and extra-murally, these afferents may also branch collaterals to myenteric and submucosal enteric ganglia (Lynn et al., 2003; Song et al., 2009; Takaki & Nakayama, 1989).

Serosal and mesenteric afferents are chemosensitive to a wide variety of algogenic mediators released during tissue damage. These may act to depolarise afferents (such as ATP, 5-hydroxytryptamine, prostaglandins, histamine and those mediators released from mast cells) and/or have a sensitising role (such as adenosine, PGE₂ and nerve growth factor) (Blackshaw & Gebhart, 2002; Grundy, 2004). Furthermore, they express receptors for inflammatory cytokines including IL-1 β , IL-6 and TNF (Andratsch et al., 2009; Binshtok et al., 2008; Y. Li et al., 2004). During experimental inflammation, the excitability of

mesenteric and serosal afferents in *ex vivo* preparations are significantly modulated (Hughes et al., 2009). This effect is mirrored *in vivo* by increased post-inflammatory visceromotor reflexes to colonic distension, which may be attributed to the sensitisation of afferent terminals by inflammatory mediators and also more long-term alterations in expression levels of relevant receptors (Gschossmann et al., 2004; Sengupta, Snider, Su, & Gebhart, 1999).

Often experimental inflammation leads to the sensitisation of afferents that were previously insensitive to mechanical stimuli (Brierley, Carter, et al., 2005; Brierley, Jones, et al., 2005; Feng & Gebhart, 2011; Feng, La, Schwartz, et al., 2012). These 'silent' or mechanically insensitive afferents constitute approximately 33% of thoracolumbar and 24% of lumbosacral spinal afferents (Feng & Gebhart, 2011). Following localised application of inflammatory soup to the receptive field, it is possible to sensitise about a quarter of silent afferents, identified in both the lumbar splanchnic nerve (LSN) and the pelvic nerve (PN), to mechanical stimuli (Feng & Gebhart, 2011). In some instances, silent afferents may remain mechanically insensitive but respond directly to chemical activation (for example, following application of bradykinin, α,β -meATP and inflammatory soup) (Brierley, Jones, et al., 2005; Feng & Gebhart, 2011). Long-term sensitisation of colonic afferents may also occur after intracolonic 2,4,6-trinitrobenzenesulfonic acid (TNBS) or zymosan treatment in mice and is associated with a persistent colorectal hypersensitivity to distension (Feng, La, Schwartz, et al., 2012; Feng, La, Tanaka, et al., 2012; Hughes et al., 2009). In both zymosan and TNBS models, there is a marked reduction in the proportions of mechanically insensitive (silent) afferents in

pelvic nerve, with a corresponding increase in the proportion of serosal afferents during the acute phases of inflammation (< 14 days after instillation) (Feng, La, Schwartz, et al., 2012; Feng, La, Tanaka, et al., 2012). This increase in the proportion of serosal afferents is also observed in the splanchnic nerve, although it is unclear whether this corresponds to the sensitisation of previously silent afferents or not (Hughes et al., 2009). The effects of chronic inflammation on afferent responses seem to vary dependent on the subtype and nerve under investigation. For example, the degree of afferent firing to von Frey probing in serosal units is reduced during the acute phase in TNBS models, but subsequently increases during the recovery period (~28 days) (Feng, La, Tanaka, et al., 2012; Hughes et al., 2009). However, responses of splanchnic muscular afferents to stretch are unaffected by TNBS-instillation (Hughes et al., 2009). This suggests that within the splanchnic nerve there exists significant plasticity in the sensitivity of serosal, but possibly not muscular afferent subtypes to inflammatory insult. It also suggests that those silent afferents that gain mechanical sensitivity post-inflammation likely represent a population of serosal afferents.

3.1.2.4 Viscerofugal enteric primary afferents

Viscerofugal enteric primary afferents project from the enteric nervous system to prevertebral ganglia and form a reflexive feedback loop from the gut to the sympathetic chain (Miller & Szurszewski, 1997). This enables feedback on gut volume to sympathetic pathways innervating the gut and marks the prevertebral ganglia as sites of coordination mediating mechano-gastrointestinal function (Szurszewski et al., 2002). Whilst these fibres are not

extrinsic in origin, a component of electrophysiological recordings from colonic nerves will be viscerofugal (Hibberd, Zagorodnyuk, Spencer, & Brookes, 2012). This will be a small proportion given that approximately 20 fibres per 5mm of colon project to prevertebral ganglia and 50% of these fibres terminate at the inferior mesenteric ganglia (IMG) (Luckensmeyer & Keast, 1995). Rectospinal neurones which originate in the myenteric plexus of the rectum and project to the spinal cord also represent a subset of viscerofugal fibres. These are thought to participate in the defecation reflex, though their physiological stimulus is not fully understood (Neuhuber et al., 1993).

3.1.3 EXPERIMENTAL STIMULATION

3.1.3.1 *In vivo* colorectal distension

The activation of pelvic pain pathways during *in vivo* animal models is monitored using pseudoaffective and visceromotor reflexes (VMR) to colorectal distension (CRD) (Ness & Gebhart, 1990). Typical experimental protocols for VMR to CRD consist of either sets of phasic balloon inflations (e.g. 15, 30, 45, 60 or 75mmHg) at 5-10 minute intervals or repetition of a single distending pressure (Shinoda et al., 2009). Responses characteristically decrease initially and then stabilise after 4-8 distensions, at this point the intervention (e.g. pharmacological agent) is applied (Kamp, Jones, Tillman, & Gebhart, 2003; Sivarao, Langdon, Bernard, & Lodge, 2007).

Evidence from decerebration indicates that visceromotor reflexes do not require higher central processing in rat, but do involve both ascending and descending pathways of the brainstem (Ness & Gebhart, 1988). This response is mediated by lumbosacral pathways in normal mouse colon with the severing of

splanchnic pathways having little effect on reflex responses (Kyloh et al., 2011). However, following inflammation thoracolumbar pathways also contribute to the VMR during CRD (Traub, 2000). To target the thoracolumbar pathways more effectively, balloon distension of the more proximal colon (30-75mm from the anus in mouse) is required. This is reflected in the more distal innervation of the lumbosacral pathway (9-15mm from the anus) (Kyloh et al., 2011). Consistent with this, in man pain evoked by distension of the colon at 16cm above the anal sphincter is mediated by the thoracolumbar pathway (Ray & Neill, 1947). By contrast pain evoked by balloon distension within 16cm of the anal sphincter was unaffected by bilateral sympathectomy (T7-L3), implicating a pelvic pathway (Ray & Neill, 1947). As such, typical VMR models applied during CRD replicate activation of pelvic pathways but poorly model thoracolumbar activation and associated behavioural responses.

Splanchnic pathways predominantly consist of serosal and mesenteric afferent fibres (>80%; (Brierley et al., 2004)), whilst pain evoked by rectal distension is likely mediated by a combination of rectal intraganglionic laminar endings (rIGLES) and rectal muscular-mucosal endings. These studies show that pain originating from the rectum is mediated by pelvic pathways, whilst more proximal pain is driven through splanchnic pathways.

Rectal distension in man evokes different sensations dependent on pressure, at low pressures this manifests predominantly as urge, and at high pressures, the sensation of pain (Kwan, Mikula, Diamant, & Davis, 2002). Further to this, in studies of patients with spinal cord lesions, slow ramp distension activates pelvic pathways with urge preceding discomfort, however painful rapid rectal

distension is mainly detected by splanchnic pathways (Lembo et al., 1994). Indeed, relaxation of the anal sphincter in rat occurs at approximately 13mmHg distending pressure, which is superseded by visceromotor responses at a threshold of 22mmHg (Ness & Gebhart, 1988). During *in vivo* rodent recordings from S1 primary afferents and from jejunal mesenteric afferents, rapid phasic distension of the bowel evoked significantly greater nerve discharge than a slow ramp distension (Booth et al., 2008; Sengupta & Gebhart, 1994). This shows that both splanchnic and pelvic pathways in rodents are activated more significantly by phasic compared to ramp distensions and suggests that differences in pain sensation experienced in man are dependent on the temporal aspect of the stimuli and are not due to differences in stimulus activation thresholds between pelvic versus splanchnic pathways. The shape of response-profiles and fibre-type frequency remains consistent whether using balloon inflation or liquid fill of the colon, however overall pelvic nerve fibre firing rate is increased (~2x) when distending by fluid (Sengupta & Gebhart, 1994; Su & Gebhart, 1998). This is also true of jejunal mesenteric afferents, where increases in responses following liquid fill of the intestine were ascribed to combined circumferential and longitudinal distortion of the bowel (Booth et al., 2008). Comparable rodent data *in vivo* on afferent response profiles to distension does not exist for the splanchnic nerve, likely due to its inaccessibility during surgical procedures.

These studies suggest that the sensations of urge and pain are mediated by functionally distinct pathways in man, through either the activation of the pelvic vs. splanchnic pathways or through the preferential activation of certain

afferent subtypes in a stimuli-dependent manner. This advocates that in rodent models, different pathways may be activated by differing distension protocols, or by the positioning of the distending balloon.

3.1.3.2 *Ex vivo* colorectal preparations

In addition to *in vivo* behavioural responses and afferent firing to noxious colorectal distension, colonic preparations have been developed *ex vivo* in a tissue bath setting. These include recordings of the pelvic and splanchnic pathways innervating the distal colon either in a tubular preparation or opened along the anti-mesenteric border in a flat-sheet preparation. Both techniques have facilitated the further study of mechanical activation of primary afferents innervating the colon, particularly to noxious stimulus. For example, intracellular recordings of colonic L6 DRG neurones in an *ex vivo* preparation combines the characterisation of high or low threshold pelvic mechanosensors with TRPV1 immunohistochemistry (Malin et al., 2009). Chemically-induced contraction of unparalysed mouse colon provides another mechanical stimulus, which may better model the partial or complete occlusion observed during diseases of the human bowel (Zagorodnyuk, Kyloh, Brookes, Nicholas, & Spencer, 2012).

In order to study the properties of individual afferent terminals, flat-sheet preparations have been used to great effect. Using direct mechanical activation of afferent receptive fields with von Frey hair, stroke or circumferential stretch, the role of key molecular pathways in mechanotransduction have been investigated, including that of ASIC, TRPV4 and TRPA1 channels (Brierley et al., 2009; Brierley et al., 2008; Page, Brierley, et al., 2005). Direct mechanical

probing of the receptive field with von Frey hair provides a highly robust and reproducible stimulus, enabling studies of mechanical sensitivity, including following application of TRPV4 ligands (ruthenium red and 5,6-epoxyeicosatrienoic acid) or TRPA1 ligands (allyl-isothiocyanate and trans-cinnamaldehyde) (Brierley et al., 2009; Brierley et al., 2008). This has also been investigated following acute application of inflammatory mediators such as bradykinin, and following chronic inflammation of the colon by TNBS (Brierley, Jones, et al., 2005; Hughes et al., 2009). The combination of functional data from afferent receptive fields, with nerve dye fills and immunohistochemistry has vastly improved the understanding of structure-function relationships of visceral afferent terminals (Lynn et al., 2003; Song et al., 2009).

Importantly, efforts have been made to understand the correlation of responses to both von Frey hair probe and circumferential stretch of receptive fields in flat-sheet preparations with *in vivo* distension or physiology of the bowel. In splanchnic nerve recordings, serosal and mesenteric afferent subtypes, identified by their relatively low sensitivity to stretch and also receptive field locale, were shown in tubular preparations to have response thresholds to distension of 53 ± 4 mmHg and 56 ± 4 mmHg, respectively (Hughes et al., 2009). The corresponding mean circumferential stretch activation threshold for serosal afferents in a flat-sheet preparation was ~ 9 g, with only a fraction recruited even at 11g of stretch. This differs significantly to the stretch activation threshold of muscular afferents in the same preparation, which respond at low threshold (< 3 g, with all recruited by 4g) (Hughes et al., 2009). This relationship between stretch and intraluminal pressure has also been

back-engineered using the formula, $\text{Pressure} = 2\pi\text{Force}/(\text{LD})$, where L is colon length and D is the circumference (Shinoda et al., 2009). In this study, 170mN ramped circumferential stretch was mathematically converted to $\sim 45\text{mmHg}$ distension (Shinoda et al., 2009). However, it is clear that neither probe by von Frey hair nor circumferential stretch, are physiological in their representation of afferent activation in the colon. Given the different sensations evoked by varying mechanical stimuli *in vivo* (e.g. urge, discomfort, pain, etc.), it seems highly likely that experimental stimuli such as von Frey hair probe or circumferential stretch will preferentially activate certain visceral pathways over others. This may lead to stimulus-dependent bias in the relative importance of a molecular pathway. In fact, these models suggest that multiple mechanical stimuli and models of visceral pain should be employed in order to understand the role of a particular pathway in painful sensation. The critical translational link is to ensure that changes in afferent terminal sensitivity or firing rates translate to behavioural differences in pain sensation in man.

3.1.4 VISCERAL MECHANOSENSITIVITY AND MECHANOTRANSDUCTION

The use of experimental models has been invaluable in our ability to understand the molecular entities responsible for mechanotransduction at the afferent terminal. This has implicated a number of mechanically-gated ion channels in visceral mechanosensation, as described in the introduction, including TRPV4, TRPA1 and ASICs. In addition to these, receptors that are mechanically-sensitive yet do not directly gate ions may trigger intracellular signalling pathways to modulate afferent terminal mechanosensitivity, such as G-protein coupled receptors angiotensin II type 1 or bradykinin B₂ receptors

(Chachisvilis, Zhang, & Frangos, 2006). Also, the overall neuronal sensitivity of the afferent terminal which is significantly regulated by inflammation will contribute to mechanosensitivity. How mechanosensitive receptors influence the function of voltage-gated ion channels and neuronal sensitivity is detailed below, with a particular focus on sodium channels and the modulatory role of Nav1.9.

3.1.4.1 Indirect regulation of mechanosensitivity

In addition to the direct gating of mechanical stimuli, the sensitivity of afferent endings to mechanosensation can be modulated by ion channels that are non-mechanically-gated, such as voltage-gated K⁺ and Na⁺ ion channels and P2X receptors. The indirect regulation of these ion channels by second-messenger pathways downstream of inflammatory receptors, act to regulate afferent baseline activity and excitability to mechanical stimuli. For example, the release of ATP following tissue damage activates P2X receptors and other purinoceptors, which can lead to increases or decreases in intestinal afferent mechanosensory function (Kirkup, Booth, Chessell, Humphrey, & Grundy, 1999; Rong, Keating, Sun, Dong, & Grundy, 2009; Wynn, Rong, Xiang, & Burnstock, 2003). Voltage-gated K⁺ ion channels contribute to the regulation of activation thresholds, firing rates and the sustained discharge of action potentials (Takeda et al., 2011). Two currents have been shown to play important roles in these functions; transient I_A type currents, likely mediated by K_V 1.4 or 4.2 in nociceptive DRG neurones, and sustained delayed rectifier (I_K) currents. The dominant channel subtype responsible for the I_K current in small DRG neurones is still to be determined but likely candidates are K_V 1.1, 1.2, 1.5, 1.6, 2.1, 2.2 and

3.1 (Choe, 2002). In a number of models of chronic visceral inflammation, including peptic ulcer, ileitis and cystitis, I_A currents are significantly reduced (Dang, Bielefeldt, & Gebhart, 2004; Stewart, Beyak, & Vanner, 2003). Further to this, I_K currents are also decreased following certain inflammatory insults, including TNBS-induced intestinal colitis in guinea-pig (Stewart et al., 2003).

As mentioned in Chapter 1, multiple voltage-gated Na^+ channels contribute to action potential electrogenesis in sensory neurones. In the viscera, total tetrodotoxin-resistant (TTX-R) Na^+ current is increased and the activation curve shifted in a hyperpolarising direction following gastric ulceration (Bielefeldt, Ozaki, & Gebhart, 2002). TNBS-induced GI inflammation evoked similar changes to TTX-R Na^+ current in DRG neurones, with no effect on tetrodotoxin-sensitive (TTX-S) Na^+ currents (Beyak et al., 2004; Stewart et al., 2003). The dominant TTX-R sodium channel current is mediated by $Na_v1.8$ and contributes to nociceptor action potential properties (Djouhri et al., 2003). The role of $Na_v1.9$ in inflammation-induced changes in TTX-R current is less defined and is discussed in more detail below. The contribution of $Na_v1.8$ current up-regulation to afferent hypersensitivity during inflammation is demonstrated by $Na_v1.8$ null mice exhibiting normal nociceptive behaviour to noxious visceral stimuli but reduced behaviours to sensitising stimuli (Laird, Souslova, Wood, & Cervero, 2002). In addition to the effects seen in chronic inflammatory *in vivo* models, acute application of inflammatory mediators, such as PGE_2 , ATP and serotonin to dissociated somatic DRG neurones can increase TTX-R Na^+ currents (Baker, 2005; Cardenas et al., 1999). This is also true for colon-

projecting neurones, where PGE₂ evoked a robust increase in TTX-R Na⁺ currents (Gold et al., 2002).

3.1.4.2 Mechanical hypersensitivity induced by artificial inflammatory soup

As such, during inflammation, afferent hypersensitivity is significantly regulated by the coordinated reduction of both I_A and I_K currents and increased TTX-R Na⁺ currents. These effects are dependent on both post-translational and transcriptional events downstream of inflammatory mediator receptor activation. Multiple mediators released during tissue damage and inflammation act in synergy to hypersensitise afferent endings and, as such, single mediators in isolation are unlikely to recapitulate an adequate stimulus in animal models (Su & Gebhart, 1998). Artificial inflammatory soups (IS), typically consisting of mediators such as bradykinin, ATP, PGE₂, histamine and 5-HT, are capable of inducing robust afferent hypersensitivity to mechanical stimuli and direct stimulation of afferent fibres. In the viscera, inflammatory soups have been used to investigate how inflammation may influence afferent function and to characterise 'silent' afferents. For example, the intracolonic instillation of IS increases resting activity and the magnitude of afferent firing to CRD in approximately 60% of fibres (Su & Gebhart, 1998). IS also reduced activation thresholds for a subset of high-threshold fibres (Su & Gebhart, 1998) and consistent with this effect, IS has also been used to sensitise 'silent' mechanically insensitive afferents, with 23% of splanchnic and 71% of pelvic afferents acquiring mechanosensitivity after application (Feng & Gebhart, 2011). Furthermore, inflammatory soups have been used to correlate *in vivo* hypersensitivity to CRD following TNBS treatment with *in vitro* afferent

hypersensitivity studies (Kiyatkin, Feng, Schwartz, & Gebhart, 2013; Tanaka et al., 2011).

3.1.4.3 Modulation of mechanical sensitivity by human tissue supernatants

Post-inflammatory hypersensitivity is a hallmark of functional bowel disorders, such as irritable bowel syndrome (IBS) (Knowles & Aziz, 2009). A subset of patients with IBS show significant visceral hypersensitivity to colorectal distension (Lembo et al., 1994; Ritchie, 1973), which is likely the result of visceral afferent modulation by low grade inflammation, release of mast cell mediators or stress hormones. Artificial inflammatory soups therefore provide a useful experimental methodology to investigate visceral hypersensitivity in animal models. In conjunction with this, supernatants derived from inflamed and normal human tissues have also provided an opportunity to modulate afferent function in a way more analogous to *in vivo* pathophysiology. Indeed, intracolonic infusion of faecal supernatants from diarrhoea-predominant IBS patients into the colorectum of mice can induce colonic hypersensitivity (Annaházi et al., 2009). Furthermore, supernatants derived from colonic mucosal biopsies of IBS patients, but not healthy controls, are capable of evoking nerve discharge from rat mesenteric afferents (Barbara et al., 2007) and causing visceral hyperalgesia in mice (Cenac et al., 2007). Multiple inflammatory mediators have been implicated in the pathogenesis of visceral hypersensitivity associated with IBS, including those released by mast cells, such as histamine, tryptase and trypsin (Annaházi et al., 2009; Barbara et al., 2007; Cenac et al., 2007).

Interestingly there appears to be some contradiction in the literature regarding visceral hypersensitivity to distension in inflammatory bowel disease (IBD). For example, remissive and quiescent ulcerative colitis (UC) and Crohn's disease (CD) patients are either normosensitive or *hyposensitive* to colorectal distension (Bernstein et al., 1996; Chang et al., 2000). This is mirrored by UC faecal supernatants evoking colonic *hyposensitivity* in mice, an effect purported to be mediated by the interplay of proteinase-activated receptor (PAR)-2 and PAR-4 signalling pathways (Annaházi et al., 2009). By contrast, IBS-like symptoms, including visceral hypersensitivity, can occur in IBD patients before disease onset, during active disease and during periods of disease quiescence (Grover et al., 2009; Isgar et al., 1983; Minderhoud et al., 2004). Whilst more evidence is required to determine the causality of changes in visceral sensitivity in IBD, the use of tissue supernatants enables this investigation to occur without bias to the constituents of an artificial inflammatory soup.

3.1.5 UP-REGULATION OF $Na_v1.9$ CURRENT BY INFLAMMATORY MEDIATORS

$Na_v1.9$ contributes a slowly inactivating persistent sodium current to the total TTX-R current observed in DRG neurones. The $Na_v1.9$ current is activated at voltages close to the resting membrane potential and is thought to regulate nerve terminal excitability (Baker et al., 2003). The $Na_v1.9$ sodium current is enhanced by inflammatory mediators such as ATP, PGE_2 , and $TNF-\alpha$, acting through G-protein coupled pathways, suggesting that $Na_v1.9$ may also play an important role in the activation of sensory nerves by inflammatory mediators (Baker, 2005; X. Chen et al., 2011; Maingret et al., 2008; Rush & Waxman, 2004). This hypothesis is supported for somatic pain by behavioural studies

demonstrating reduced hypersensitivity to inflammatory stimuli in rodents where Nav1.9 has been deleted or knocked down (Amaya et al., 2006; Lolignier et al., 2011; Priest et al., 2005).

Whilst the presence of Nav1.9 has been reported in visceral afferents (Beyak et al., 2004; Hillsley et al., 2006), evidence for a role of Nav1.9 in visceral pain processing is controversial, with behavioural studies in Nav1.9 knock-out mice reporting either reduced or enhanced pain behaviours to noxious stimuli (Leo et al., 2010; Martinez & Melgar, 2008; Ritter et al., 2009). The contribution of Nav1.9 to the sensitivity of visceral afferent endings to noxious stimuli thus remains unclear. Given the lack of information of afferent responses to differing mechanical paradigms from the splanchnic nerve in mouse, there is a necessity to define the mechanosensitivity of splanchnic afferents and to understand the role of Nav1.9 in these subtypes.

3.2 AIMS

1. To investigate the effects of Nav1.9 gene deletion in mouse on the response of visceral afferents to mechanical distension of the intact colon
2. To characterise mechanosensory function in afferent subtypes of the colonic splanchnic pathway in Nav1.9 -/- mice
3. To investigate the effects of inflammatory soup on direct excitation and mechanical hypersensitivity of afferent subtypes in Nav1.9 -/- mice
4. To investigate the effect of Nav1.9 deletion on afferent excitation and mechanical hypersensitivity to human-derived inflammatory bowel disease supernatants

3.3 METHODS

All experimental studies were performed in accordance with the UK Animal (Scientific Procedures) Act 1986. Human tissue was collected and used with approval of the East London and The City HA Local Research Ethics Committee (NREC 10/H0703/71).

3.3.1 *Nav1.9* KNOCK-OUT MICE

Nav1.9 $-/-$ mice were re-derived from *Nav1.9* $+/-$ breeding pairs and originally generated by homologous recombination on a C57/BL6 background as described previously (Ostman et al., 2008). The *Nav1.9* knock-out was generated by replacing exons 4 and 5 of the *SCN11A* gene with a neomycin resistance cassette at intronic restriction sites. This removed the domain I, S4 voltage sensor from the translated *Nav1.9* protein. Ostman *et al.* confirm the subsequent knock-out allele produced a mutant mRNA lacking exons 4 and 5. Specifically, through cDNA sequencing of the transcript they show that the splicing machinery skips the neomycin selection cassette and splices exon 3 to exon 6. During translation this leads to a frameshift mutation which results in the introduction of stop codons in exon 6 (Ostman et al., 2008). *Nav1.9* KO mice were healthy and fertile (Ostman et al., 2008). An alternate *Nav1.9* KO strain (B6.129P2-*Scn11^{atm1Dgen}*/J), commercially available from The Jackson Laboratory mouse repository, does not possess significant differences in lifespan, fertility, blood chemistry, length or weight (Priest et al., 2005). Furthermore necropsy and histology did not differ between genotypes. *Nav1.9* $-/-$ mice and *Nav1.9* $+/-$ littermate controls were initially derived from a

heterozygous mutant (+/-) x heterozygous mutant (+/-) breeding pair strategy. Mice generated from these pairings were primarily used during pilot studies, early experiments refining protocols and in the establishment of subsequent pairings. Approximately four wild-type (+/+) x wild-type (+/+) and four knock-out (-/-) x knock-out (-/-) breeding pairs were established and maintained during subsequent studies, typically producing litters of 4-9 pups every 6 weeks. The divergence of genetic lines (genetic drift) and likelihood of development of inbred sub-strains was limited by ensuring mutants were backcrossed onto the parental inbred strain every 10 generations (in this case standard C57BL/6 strain) (J. G. Fox, 2007). All mice used in these studies were within 10 generations from any heterozygous mutant (+/-) x heterozygous mutant (+/-) pairing. Mice were maintained in a 12 hour light/dark cycle and had *ad libitum* access to food and water. Pinna biopsies were obtained at weaning (21 days) for use in genotyping.

3.3.1.1 Genotyping

Pinna biopsies were lysed in 500µl lysis buffer (0.1M Tris-HCl pH 8.5, 5mM ethylenediaminetetraacetic acid (EDTA), 0.2% sodium dodecyl sulphate (SDS), 20mM NaCl, 50µg proteinase K) for 6 hours at 50°C. Non-digested organic material was pelleted by brief centrifugation and 500µl of isopropanol added to the supernatant. The supernatant was subsequently centrifuged at 4°C for 15 minutes followed by addition of 500µl 70% ethanol. After a final centrifugation at 4°C for 10 minutes, the supernatant was removed and the pellet allowed to dry overnight. Re-suspension of the extracted genomic DNA in 40µl sterile water enabled quantification of DNA concentration by Nanodrop

spectrophotometer (Thermo Scientific, UK). Approximately 50-70ng of DNA was added to two 10µl PCR reactions (HotStar TAQ PCR Master Mix, Qiagen). A common 5' primer was used for both reactions (5'- ATGTGGCACTGGGCTTGAAGTC - 3'), whilst the 3' primer was either specific for the deleted exons 4 and 5 (wild-type: 5' – AACAGTCTTACGCTGTTCCGATG - 3') or the inserted neomycin gene (mutant: 5'- CTCGTCGTGACCCATGGCGAT - 3'). The reaction used was 35 cycles of 95°C for 1 minute, 57°C for 1 minute and 72°C for 2 minutes, with a preceding 15 minute hot-start at 95°C. PCR reactions were subsequently separated by gel electrophoresis on a 1% agarose gel containing 0.5% ethidium bromide or RedSafe nucleic acid stain (Chembio, UK) at 100V for 25 minutes. Under UV illumination, a band at 300 base pairs (bp) was visible for the wild-type gene and at 600 bp for the mutated gene.

3.3.2 IN VITRO MOUSE COLONIC SPLANCHNIC AFFERENT PREPARATIONS

Adult Nav1.9 +/+ or Nav1.9 -/- mice of either sex were euthanised by rising concentration of CO₂ and the distal colon with associated lumbar splanchnic nerves removed. Specifically the distal colon from the ascending/descending inflexion point to anus, inclusive of rectum, was removed. Simultaneously, the skeletal muscle and neurovascular bundles (including the aorta) were cut ventrally from the spinal column whilst retaining the inferior mesenteric artery and lumbar splanchnic innervation of the colon. The lumbar colonic nerve was then traced into the aortic neurovascular bundle at the point of the iliac bifurcation, and the inferior and superior mesenteric ganglia identified (see Figure 17C). Suction electrode recordings were made from one of the two intermesenteric nerves between these two ganglia, rostral to the inferior

mesenteric ganglion (see Figure 17D). For whole-nerve experiments, tissues were cannulated, lumenally perfused (100µl/min) and serosally superfused (7ml/min; 32-34°C) with carbogenated Kreb's buffer (in mM: 124 NaCl, 4.8 KCl, 1.3 NaH₂PO₄, 2.5 CaCl₂, 1.2 MgSO₄·7H₂O, 11.1 glucose, and 25 NaHCO₃) supplemented with nifedipine (10 µM) and atropine (10 µM) to block smooth muscle contraction, and indomethacin (3 µM) to reduce on-going inflammation (see Figure 17A). For teased-fibre recordings, tissues were treated similarly, however the colon was opened along the anti-mesenteric border and pinned flat mucosal side up (see Figure 17B), as described previously (Brierley et al., 2004; Brierley et al., 2008). Extraneous skeletal muscle and connective tissues were removed from the neurovascular bundle allowing unrestricted access to the intermesenteric nerve. Using fine forceps, this bundle was teased apart into 5-8 fibres for electrophysiological teased-fibre recordings.

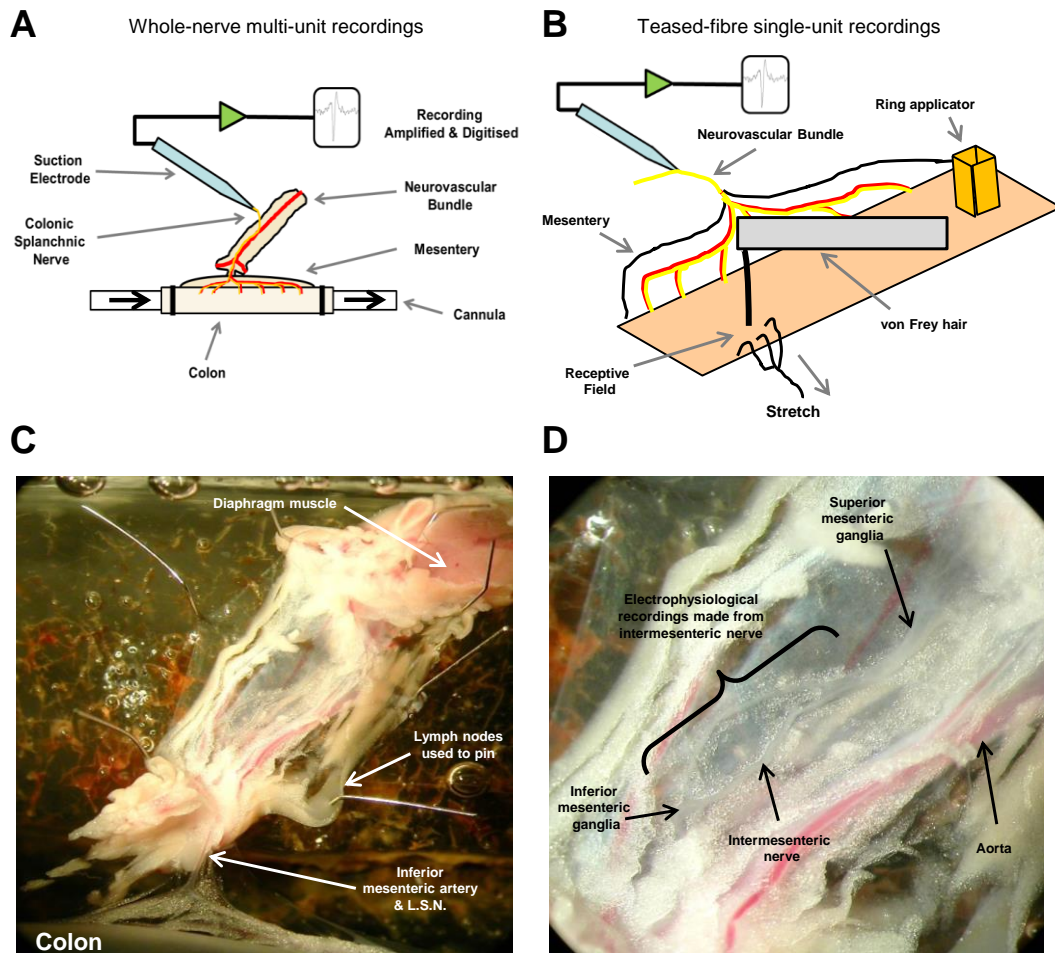


Figure 17. Schematics of the two electrophysiological recording techniques used to investigate visceral afferent function. A Whole-nerve multi-unit recording of lumbar splanchnic nerve activity. The colon was cannulated and perfused in an oral to aboral direction. The lumbar splanchnic nerve was dissected free and whole-nerve suction electrode recordings made of nerve activity. B Teased-fibre recordings were performed by opening the colon along the anti-mesenteric border and pinning the colon flat. The lumbar splanchnic nerve was teased apart under microscopic dissection into 5-8 teased fibres. These were individually isolated in the suction electrode and single receptive fields characterised by von Frey probe, circumferential stretch or light stroke. C Photo of the dissection pinned in the recording chamber. Along the bottom the colon and mesentery can be seen, with the inferior mesenteric artery and lumbar splanchnic nerve (LSN) entering the aortic neurovascular bundle at the iliac bifurcation. Typically six pins were used to secure the bundle ventral side up during blunt dissection of the intermesenteric nerve. D Close-up photo of inferior and superior mesenteric ganglia during electrophysiological recordings.

3.3.3 ELECTROPHYSIOLOGICAL RECORDINGS AND CHARACTERISATION OF COLONIC SPLANCHNIC AFFERENT PROPERTIES

Multi-unit activities were recorded from whole lumbar splanchnic nerves (rostral to the inferior mesenteric ganglia) or single-fibre activities were recorded from teased lumbar splanchnic nerves using a borosilicate glass suction electrode. Signals were amplified, band pass filtered (gain 5K; 100-1300 Hz; Neurolog, Digitimer Ltd, UK) and digitally filtered for 50Hz noise (Humbug, Quest Scientific, Canada). Raw traces were digitised at 20 kHz (micro1401; Cambridge Electronic Design, UK) and action potential firing counts were determined using a threshold of twice the background noise (typically 100 μ V). All signals were displayed on a PC using Spike 2 software. In multi-unit tubular preparations, the colon was tied, using fine thread, to two cannulas, and perfused with Kreb's buffer at 0.1ml/min (Figure 17A). The pressure was set to baseline and an end pressure of ~2mmHg applied before initiating the experimental protocols. Preparations were left for approximately 45 minutes or until stable baseline was observed. In teased-fibre recordings from flat sheet preparations (Figure 17B), individual teased nerve bundles were brought into the suction electrode and nerve activity assessed. If none, or almost none, ectopic nerve discharge was observed, then a mechanical search strategy was employed to identify receptive fields. Specifically, gentle application of a cotton bud was systematically used to stimulate the mesentery and mucosal surface of the colon wall. Where action potentials were elicited by mechanical probe then distinct receptive fields were identified and characterised according to previously published classifications, detailed below (Brierley et al., 2004; Harrington et al., 2011; Hughes et al., 2009). Ideal recordings possessed no

more than a single mechanically sensitive action potential waveform, however where ectopic background activity was present, minimal (<3) action potential waveforms should be observed and these should be easily distinguishable from the waveform of interest. This receptive field was subsequently characterised by graded stimulus-response to punctate von Frey hair (vFh) probing (0.07g, 0.16g, 0.4g, 1g and 2g; each applied 3 times for a period of 3s), circumferential stretch (0g, 5g & 10g; each weight applied for 1 min, with an interval of 1 min between applications) and mucosal stroking with light vFh (0.16g; applied 10 times). A cantilever system was used to apply stretch via a thread attached with a purpose-made claw to the tissue adjacent to the receptive field. Addition of weights to the end of the cantilever system initiated colonic stretch. Four distinct classes of afferent fibre were identified based on these responses: muscular (those responding to low intensity circumferential stretch [≤ 5 g], but not fine mucosal stroking); mucosal (those responding to light vFh stroking), mesenteric (those responding to focal compression of the mesentery) and serosal (those responding to focal compression of the colon wall, but not low intensity stroke or low intensity circular stretch. Recent literature suggests that 'serosal' and 'mesenteric' afferent classes have their transduction sites on, or close to, blood vessels, including submucosal vessels, in guinea-pig, and that classification as 'vascular' may be more appropriate (Brookes et al., 2013; Hughes et al., 2009; Song et al., 2009). In the absence of detailed anatomical classification in mouse and to provide continuity with existing studies, the current nomenclature of serosal and mesenteric afferent endings will be used for the purposes of this study. In addition, conduction velocity was calculated for serosal and mesenteric units by dividing evoked action potential latency

elicited following electrical stimulation (0.5Hz, 15V, 1ms) of the receptive field with concentric stimulating electrodes, by the distance from the recording electrode to receptive field. This also confirmed the single fibre nature of the recording, correlating the receptive field to the evoked action potential. Typically, identified receptive fields in the mesentery and colonic wall were highly localised ($<1\text{mm}^2$), however in some instances, mesenteric or non-stretch responsive colon wall units could be stimulated in multiple (2-3) distinct and discontinuous receptive fields over an area of $\sim 15\text{mm}^2$. Where such multiple areas of stimulation for a single action potential waveform were identified then the characterisation and subsequent chemical challenge was all performed on one such receptive field area.

3.3.4 GENERATION OF HUMAN TISSUE SUPERNATANTS

Resected human colon was obtained after full written consent from patients undergoing elective surgery at Barts Health NHS Trust, London after approval by the local Research Ethics Committee (NREC 10/H0703/71). Macroscopically normal colon ($> 10\text{cm}$ from tumour) was obtained from patients undergoing colectomy as part of their normal surgical treatment for bowel cancer, whilst chronically inflamed colon or intestine was obtained from patients undergoing operations as part of their standard surgical treatment for Crohn's disease or ulcerative colitis (see Table 6). Tissue samples were subsequently incubated in fresh carbogenated Krebs' buffer at 37°C for 25 minutes at a fixed volume of 2.5ml/g of tissue. Following incubation, the tissue was removed and the buffer centrifuged at 2000g for 10 minutes. The remaining supernatant was aliquoted and stored at -80°C until use.

#	Disease	Operation	Age	Sex	Cytokine analysis	Whole nerve	Teased fibre
1	Cancer	Laparoscopic anterior resection	50	F	Y	Y	
2	Cancer	Right hemicolectomy	76	M	Y	Y	
3	Cancer	Subtotal colectomy	57	M	Y	Y	
		<i>Mean age / M:F ratio</i>	<i>61</i>	<i>2:1</i>			
4	Crohn's Disease	Right hemicolectomy	21	F	Y	Y	Y
5	Crohn's Disease	Redo right hemicolectomy	30	F	Y	Y	Y
6	Crohn's Disease	Right hemicolectomy	21	M	Y	Y	
7	Crohn's Disease	Completion colectomy	25	M		Y	
8	Crohn's Disease	Subtotal colectomy	30	M			Y
9	Crohn's Disease	Small bowel resection	24	F			Y
10	Crohn's Disease	Small bowel resection	42	M			Y
11	Crohn's Disease	Colectomy & end Ileostomy	28	M			Y
12	Crohn's Disease	Extended right hemicolectomy	16	M			Y
13	Crohn's Disease	Subtotal colectomy	64	M			Y
		<i>Mean age / M:F ratio</i>	<i>30</i>	<i>2.3:1</i>			
14	Ulcerative colitis	Subtotal colectomy	21	M	Y	Y	
15	Ulcerative colitis	Restorative proctocolectomy	20	M	Y	Y	
16	Ulcerative colitis	Subtotal colectomy	30	M	Y	Y	
17	Ulcerative colitis	Restorative proctocolectomy	18	M	Y	Y	
		<i>Mean age / M:F ratio</i>	<i>22</i>	<i>4:0</i>			

Table 6. Patient details of human tissue used in the present studies. Supernatants generated from these tissues may have been used for inflammatory cytokine quantification (Cytokine analysis), 20mL bath superfusion of whole-nerve recordings from the lumbar splanchnic nerve (whole-nerve) or discrete ring application to characterised receptive fields in a flat-sheet preparation and single-fibre recording from the lumbar splanchnic nerve (teased-fibre).

3.3.5 INFLAMMATORY CYTOKINE QUANTIFICATION

Quantitative analysis of protein cytokine levels was performed on supernatant samples using capture sandwich immunoassay and magnetic microsphere methodology. Samples were prepared as per manufacturer's instructions (Invitrogen, UK) and analysed using the Luminex MAGPIX detection system (Luminex, TX, USA) for IL-1 β , IL-6, GM-CSF, TNF- α and IL-8.

3.3.6 PROTOCOLS FOR TUBULAR PREPARATION OF MOUSE COLON

For ramp distensions, luminal outflow cannula was blocked and a subsequent increase in pressure observed, until the desired maximum pressure was reached (e.g. 80 or 145mmHg). It took approximately 4-5 minutes to reach 145mmHg. In some instances, ramp distensions occurred following a return to baseline activity after drug application. Sets of six, rapid phasic ramp distensions (0-80mmHg, 60sec at 9 minute intervals) were implemented in separate experiments. In multi-unit experiments, supernatants were applied after a stabilising period by bath superfusion of a 20ml volume. Where repeat concentrations of drugs were given, a minimum 60 minute interval was maintained. Supernatants derived from Crohn's disease (CD), ulcerative colitis (UC) and control tissues were applied by 20ml bath superfusion in separate colonic preparations. In separate experiments, mechanical sensitisation of responses to noxious ramp distensions was investigated by intraluminal perfusion of an inflammatory soup (consisting of 1 μ M bradykinin, 1 mM ATP, 10 μ M histamine, 10 μ M prostaglandin E₂ and 10 μ M 5HT) 20 minutes prior to, and during, subsequent 0-80mmHg ramp distension.

3.3.7 PROTOCOLS FOR FLAT-SHEET PREPARATION MOUSE COLON

In single-fibre experiments, receptive fields were identified by systematic probing of the colon wall and mesentery. Once identified, receptive fields were characterised as described above by location and response to mucosal stroking and circumferential stretch. Only muscular, mesenteric and serosal receptive fields were investigated in this study. In separate experiments, mesenteric and serosal units were probed by 2g von Frey hair, followed by placement of a small brass ring over the receptive field. The ring contents were aspirated and either an inflammatory soup (consisting of 1 μ M bradykinin, 1 mM ATP, 10 μ M histamine, 10 μ M prostaglandin E₂ and 10 μ M 5HT; 2 minutes) or Crohn's Disease inflammatory supernatant (see above; 5 minutes) applied. After which the ring was once again aspirated, removed and the receptive field re-probed by 2g von Frey hair.

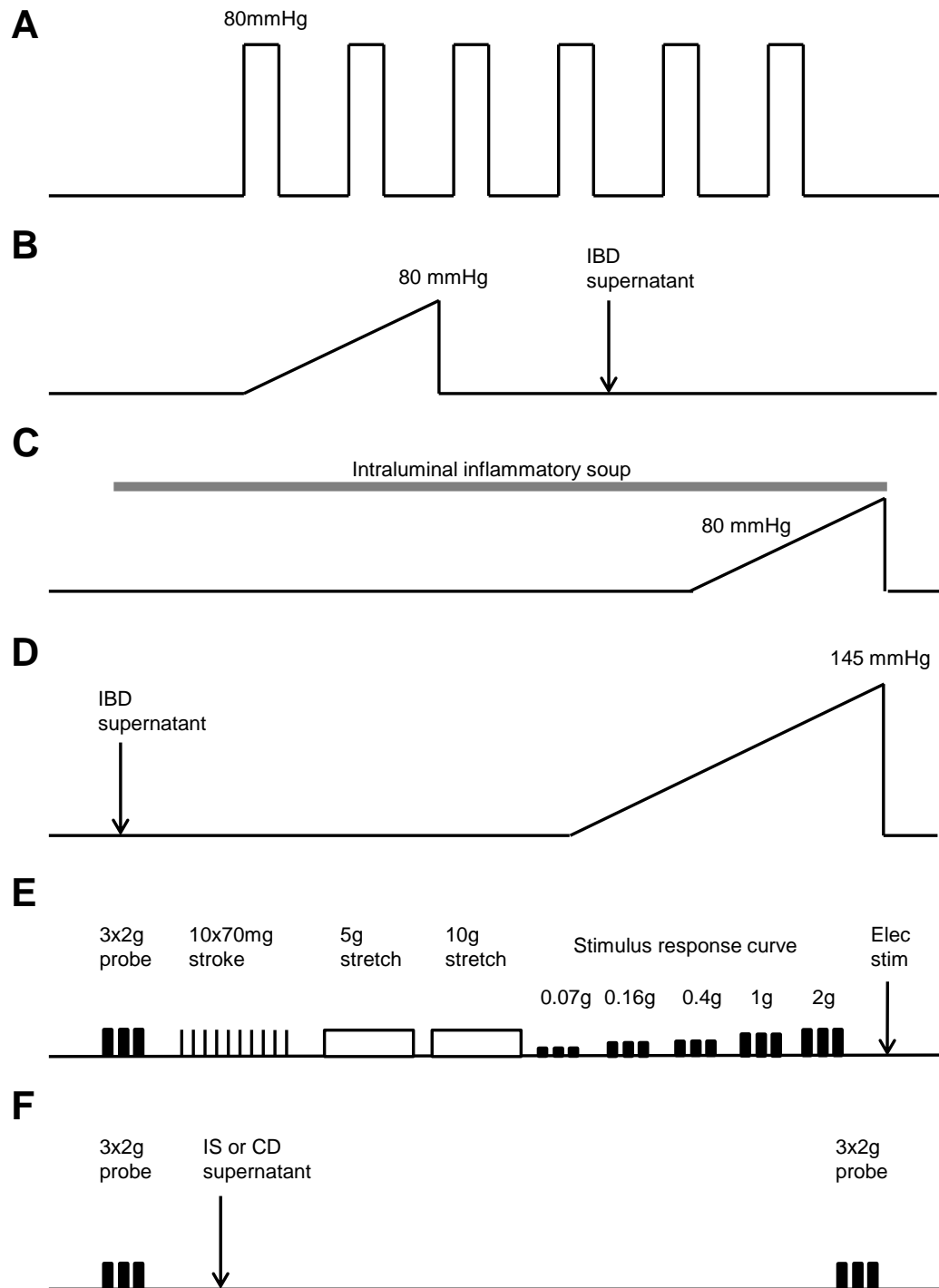


Figure 18. Diagram of protocols used during whole-nerve and teased fibre recordings. Whole-nerve recordings were made using protocols A, B, C and D. A Six rapid phasic distensions to 80mmHg for 60 sec at 9 minute intervals. B Slow ramp distension to 80mmHg followed by superfusion of 20ml IBD supernatant. C Pre-treatment with intraluminal perfusion of inflammatory soup (20mins) and during ramp distension to 80mmHg. D Application of 20ml IBD supernatant followed by slow ramp distension to 145mmHg. E Protocol for characterising receptive fields

during teased fibre recordings; 3x2g probes at 3 sec intervals, 10x70mg strokes, stretch for 60 sec (5g and 10g), stimulus response curve (3x 0.07g, 0.16g, 0.4g, 1g, 2g probes are 60 sec intervals), electrical stimulation (0.5Hz, 15V, 1ms). F Once characterised, 3x2g probes were applied before and after application of either inflammatory soup (IS) or Crohn's Disease (CD) supernatant for 2 minutes or 5 minutes, respectively, by ring application over the receptive field.

3.3.8 DATA ANALYSIS

In multi-unit experiments, peak changes and time profiles of electrophysiological nerve activity were determined by subtracting baseline firing (3 minutes before distension) from increases in nerve activity following distension. Changes in nerve activity were compared between Nav1.9 $+/+$ and Nav1.9 $-/-$ animals using Student's t-test or two-way ANOVA with Bonferroni's post-hoc test, as appropriate. During phasic distensions, peak firing was determined as the maximal firing rate observed during the distension, typically within 10 seconds of pressure increasing. Plateau firing was determined as the firing rate at 45 seconds after pressure increasing. For post-hoc single unit analysis of 0-145mmHg ramp distensions, multi-unit splanchnic nerve recordings contained action potentials that were discernibly different in amplitude and waveform to enable accurate discrimination from each other using the waveform analysis function of CED Spike 2.0 software, based on previous methodologies (Hillsley, Kirkup, & Grundy, 1998). Individual waveform templates were derived using those spikes with amplitude greater than the set threshold ($\sim 100\mu\text{V}$). The action potential waveforms were corrected for DC offset, averaged and assigned to waveform templates. Spikes were either matched to a waveform template or left unclassified depending on strict sorting criteria; specifically amplitude error was set to 3% and at least 95% of points had to fall within the template. The pressure activation thresholds and average firing frequency of individually identified units were determined). With these strict criteria, between 5-16 units were identified per

recording, which were subsequently confirmed manually by eye to ensure the quality of spike discrimination.

Response profiles of individual units were used to classify the unit as either low-threshold (LT), wide dynamic range (WDR) or high-threshold (HT). To enable objective classification, a comparable methodology to that used by Booth *et al.* was employed (Booth et al., 2008). The relative firing at low and high distending pressures was determined by expressing the response at 30mmHg as a percentage of the response at 80mmHg. Specifically, a linear increase in afferent discharge gives a percentage of 37.5%. A response profile with a value above this represents greater firing in the low distending range compared to higher pressures, and values below this respond at high distending pressures. As such response profiles with a ratio of $< 15\%$ were considered HT units and those with a ratio of $> 55\%$ were considered LT units. Afferents between these two values were considered to have a more linear increase in afferent firing and classified as WDR units (Examples can be seen in Figure 2).

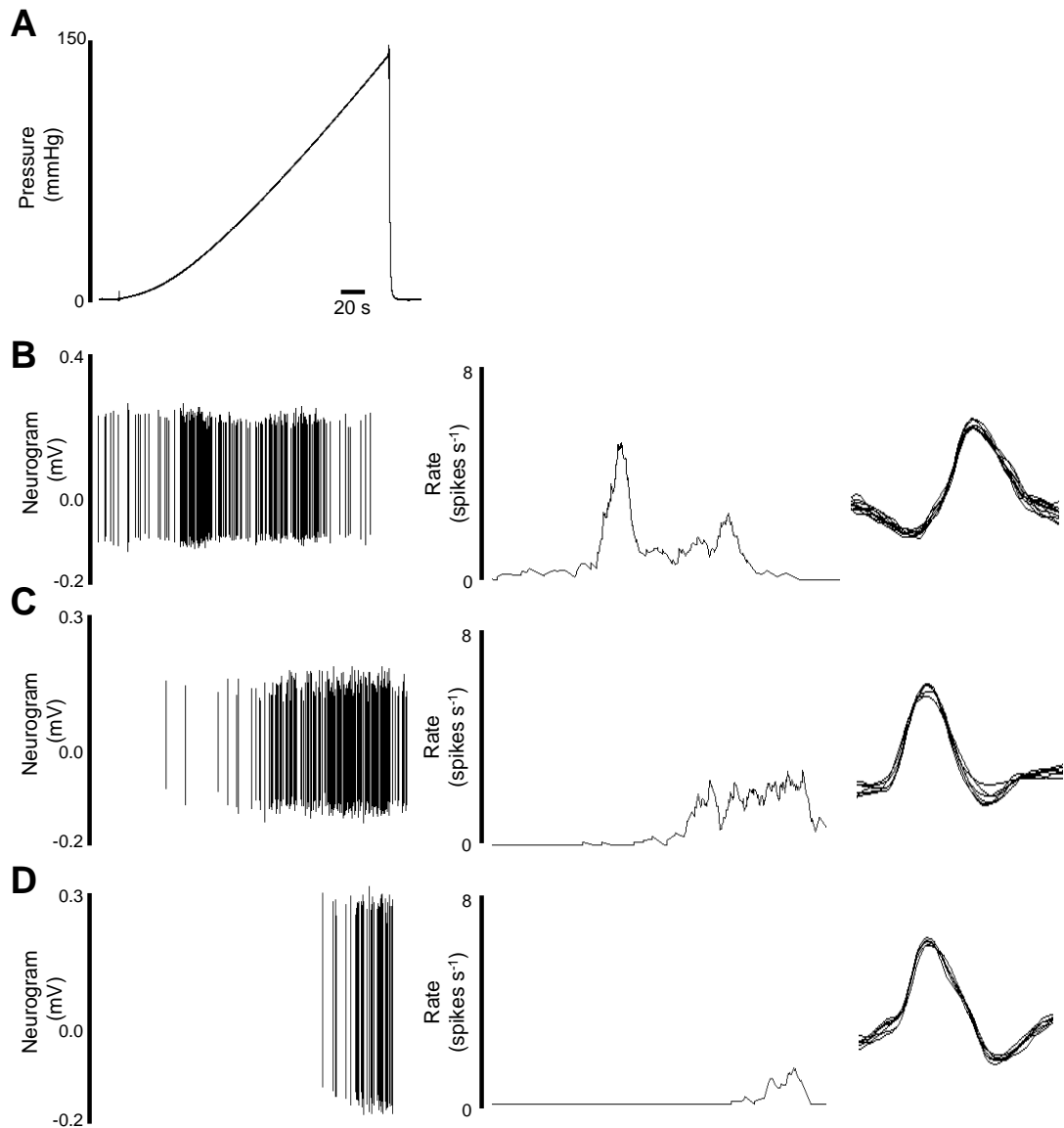


Figure 19. Example of post-hoc single unit analysis from multi-unit recording made during ramp distension. A Pressure trace of ramp distension to 145mmHg. Single unit discrimination based on action potential amplitude and duration was used to create waveform templates. All spikes above threshold matching this template were considered to represent the same single unit. Example firing rates from three units with overlays of 5 action potentials are shown in B, C and D. Specifically, B is an example of a low-threshold unit, C is an example of wide dynamic range unit and D is an example of a high-threshold unit. Action potential template matching was very robust and reliable.

In teased-fibre recordings from flat sheet experiments, average spikes/s per stimulus were compared between genotype or before and after ring application of drugs/supernatants. All cytokine quantification data were analysed non-parametrically. If detectable levels of cytokines were present in control supernatants, Mann-Whitney U-tests were performed, or else Wilcoxon Signed Rank tests comparing to a theoretical median of 0.0 were performed. Statistical significance was set at $p < 0.05$. Data have been displayed as mean \pm SEM; N = number of animals and n = number of observations.

3.3.9 DRUGS

Stock concentrations of ATP (300mM; water), PGE₂ (1mM; ethanol), bradykinin (10mM; water), histamine (100mM; water), 5HT (10mM; water), atropine (10mM; ethanol), indomethacin (3mM; DMSO) and nifedipine (10mM; DMSO) were all purchased from Sigma Aldrich (UK) and prepared as described. Inflammatory soup (IS) was prepared in advance and aliquots frozen until use. All compounds were diluted to working concentrations in buffer on the day of experimentation.

3.4 RESULTS

3.4.1 DELETION OF $Na_v1.9$ INCREASES MECHANOSENSORY ACTIVATION THRESHOLDS OF AFFERENT FIBRES AND REDUCES MAINTENANCE OF REPEATED RESPONSES

How $Na_v1.9$ influences mechanosensation was first investigated using whole-nerve recordings of distension responses in a cannulated tubular preparation of the distal colon, and subsequently using single-fibre recording techniques in a flat sheet preparation. Phasic distension of the bowel to 80mmHg intraluminal pressure led to a robust initial increase in afferent activity, subsequently reaching a plateau for the remainder of the 1 minute distension (Figure 20C). Second and third repeat distensions (at 9 minute intervals) evoked reduced peak responses, until a stabilised peak response was reached by the fourth to sixth distension. In $Na_v1.9^{-/-}$ mice, peak responses to first distension were similar to control animals ($p = 0.17$, Figure 20C). However, afferent responses to subsequent repeat distension in $Na_v1.9^{-/-}$ mice showed greater tachyphylaxis with significant reductions observed by the third to sixth distensions ($p < 0.001$, Figure 20E). These deficits appear more pronounced when examining the plateau phase of the distension, with reductions in firing rates seen during the first distension and significant differences seen in all subsequent distensions between genotypes (Figure 20E).

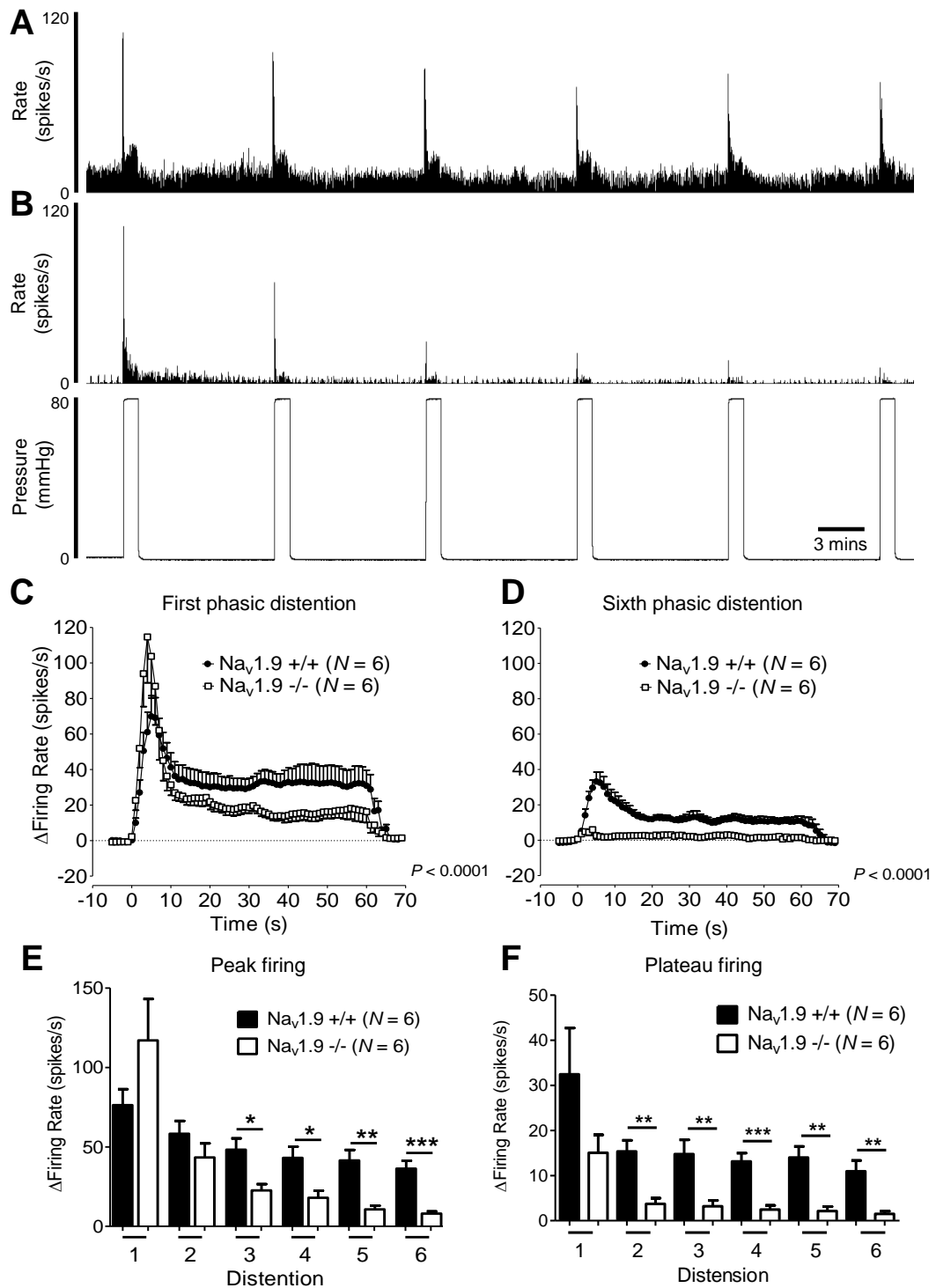


Figure 20. Colonic splanchnic nerve responses to repeat phasic distention. Example rate histogram of colonic splanchnic nerve response to repeat phasic distention (0-80mmHg; 60s; 10min intervals) in a $Na_V1.9^{+/+}$ (A) and $Na_V1.9^{-/-}$ mice (B). Average response profiles to the 1st (C) and 6th (D) phasic distention in $Na_V1.9^{+/+}$ and $Na_V1.9^{-/-}$ mice (2-way ANOVA). E Peak change in firing rate during sequential phasic distentions (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, unpaired t-test). F Change in firing rate during plateau phase (time = 45s) of sequential phasic distentions (** $p < 0.01$; *** $p < 0.001$, unpaired t-test).

These findings suggested a particular role for Nav1.9 in responses to persistent stimuli. To investigate this role further a slow ramp distension paradigm was used. Initial ramp distensions up to 80mmHg intraluminal pressure evoked maintained responses with an almost linear correlate between afferent activity and pressure, reaching a maximum firing rate of 13.3 ± 3.7 spikes/s ($N = 6$) in control animals. Nav1.9 $-/-$ mice, by contrast, elicited virtually no activity during comparable ramp distensions (max. 1.4 ± 0.6 spikes/s, $N = 6$, $p < 0.0001$, Figure 21C). Mechanical sensitisation of firing evoked by noxious ramp distension was investigated by intraluminal perfusion of inflammatory soup (IS). In Nav1.9 $+/+$ mice, perfusion of IS led to direct activation of basal afferent firing rates, which was not observed in Nav1.9 $-/-$ mice (3.8 ± 0.6 spikes/s, $N = 5$ vs. 0.0 ± 0.4 spikes/s, $N = 6$; $p < 0.001$, unpaired t-test). Responses to ramp distension during IS perfusion were potentiated in Nav1.9 $+/+$ mice (max. 19.4 ± 2.7 spikes/s, $N = 6$), an effect not observed in Nav1.9 $-/-$ up to 50mmHg (Figure 21C). At greater ramp distension pressures, sensitized responses were observed in Nav1.9 $-/-$ tissues compared to control experiments (max. 6.5 ± 1.9 spikes/s, $N = 6$), which may reflect either the sensitisation of afferent fibres negative for Nav1.9 or recruitment of alternative sensitising mechanisms within Nav1.9 containing fibres.

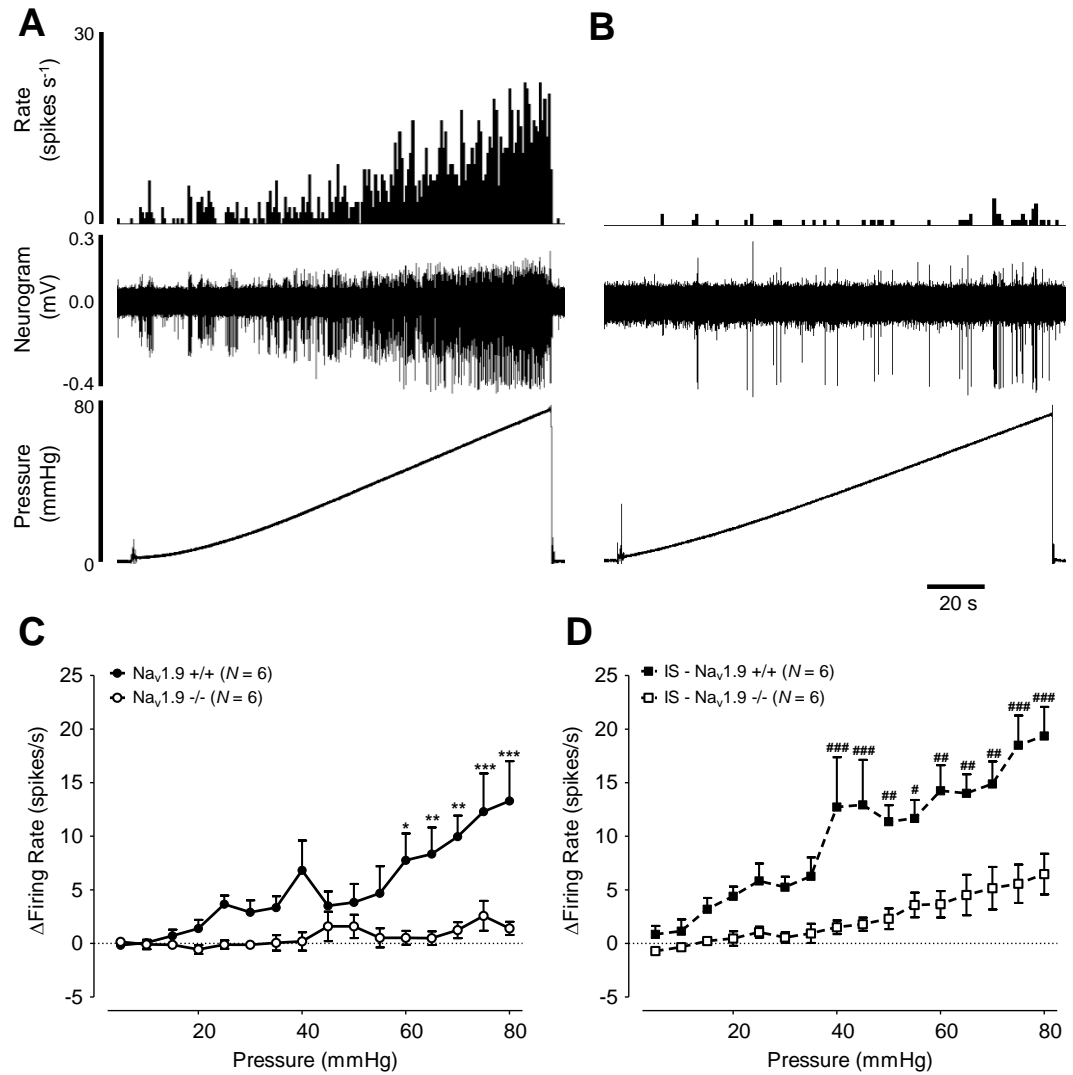


Figure 21. Colonic splanchnic nerve responses to 0-80mmHg ramp distension. Example rate histogram and raw trace of colonic splanchnic nerve response to ramp distension (0 to 80mmHg) in $Na_V1.9$ $+/+$ (A) and $Na_V1.9$ $-/-$ (B) mice with intraluminal pressure trace. Average response to ramp distension (0 to 80mmHg) in $Na_V1.9$ $+/+$ and $Na_V1.9$ $-/-$ mice in the absence (C) or presence (D) of intraluminal perfusion with inflammatory soup (IS) ($p < 0.0001$, $Na_V1.9$ $+/+$ vs. $Na_V1.9$ $-/-$, 2-way ANOVA with Bonferroni's post-hoc, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $p < 0.0001$, $Na_V1.9$ $+/+$ in presence of IS vs. $Na_V1.9$ $-/-$ in presence of IS, 2-way ANOVA with Bonferroni's post-hoc, $\#p < 0.05$, $##p < 0.01$, $###p < 0.001$).

It was reasoned that rapid phasic distension in the experiments described above develops a more substantial stimulus to mechanoreceptive afferents compared to a slow ramp fill, even up to equivalent final pressures (80mmHg), something that has been shown in both pelvic and mesenteric nerves previously (Booth et al., 2008; Sengupta & Gebhart, 1994). Therefore, it should be possible to overcome the lack of response to slow ramp distension by increasing the stimulus to supra-physiological distension pressures. The maximum distension pressure achievable in mouse colon before rupture was determined at 159.6 ± 4.3 mmHg ($N = 10$). Ramp distension to 145mmHg was thus used to provide a stronger stimulus. Ramp distensions beyond 80mmHg continued to linearly increase afferent firing rates to a maximum of 34.6 ± 7.3 spikes/s in Nav1.9 +/+ mice (Figure 22C). However, in Nav1.9 -/- mice, afferent firing remained just above baseline (<2 spikes/s) up to ~ 95 mmHg, above which an exponential increase in firing was observed reaching firing rates almost comparable to Nav1.9 +/+ animals at 145mmHg (25.4 ± 7.8 spikes/s, Figure 22D).

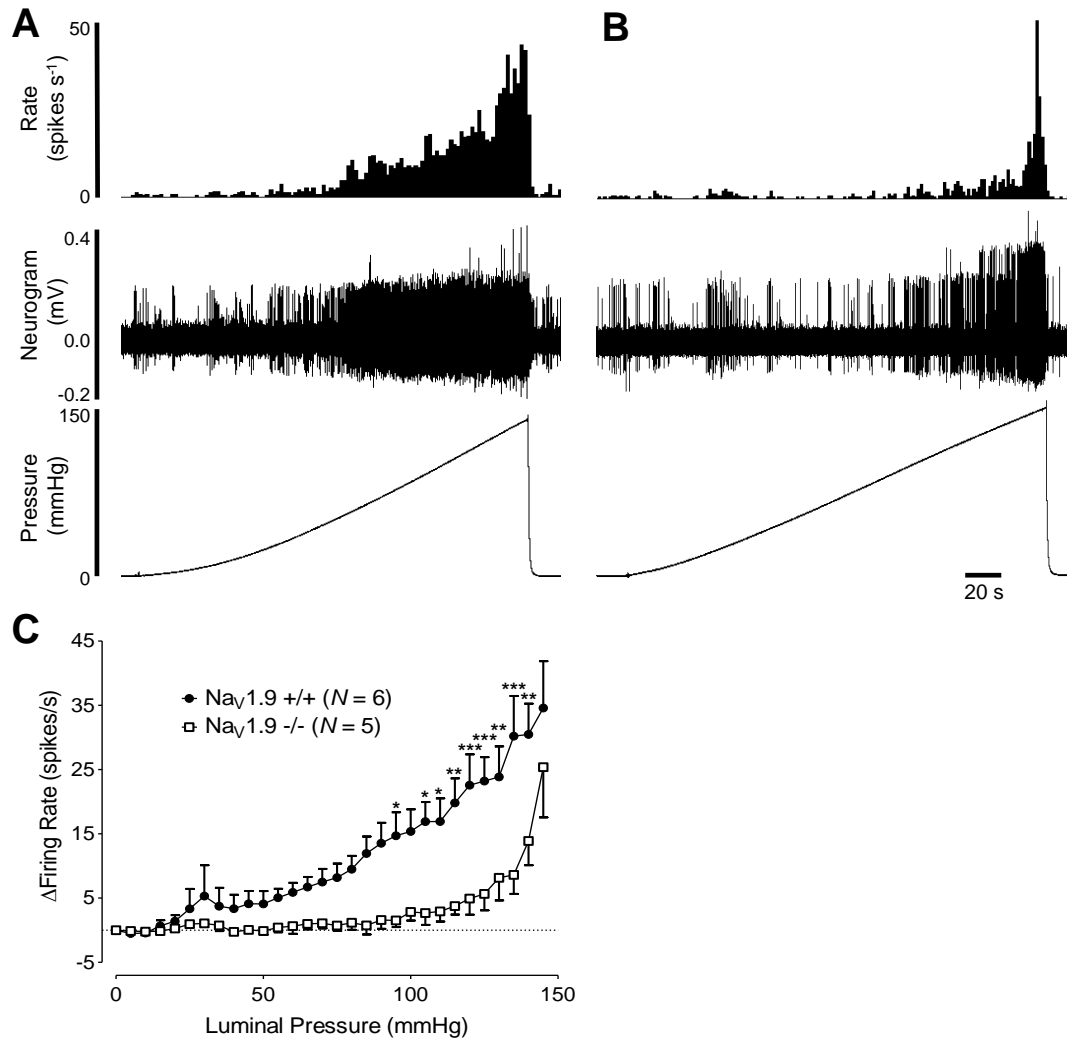


Figure 22. Colonic splanchnic nerve responses to 0-145mmHg ramp distension. Example rate histogram and raw trace of colonic splanchnic nerve response to ramp distension (0 to 145mmHg) in $Na_V1.9$ +/+ (A) and $Na_V1.9$ -/- (B) mice with intraluminal pressure trace. C Average response to ramp distension (0 to 145mmHg), firing rates to distension pressures of <95mmHg were greatly attenuated in $Na_V1.9$ -/- animals compared to $Na_V1.9$ +/+ mice ($N=6$), with an increase in firing at greater pressures in $Na_V1.9$ -/- mice ($p < 0.0001$, 2-way ANOVA with Bonferroni's post-hoc, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Single unit response profiles

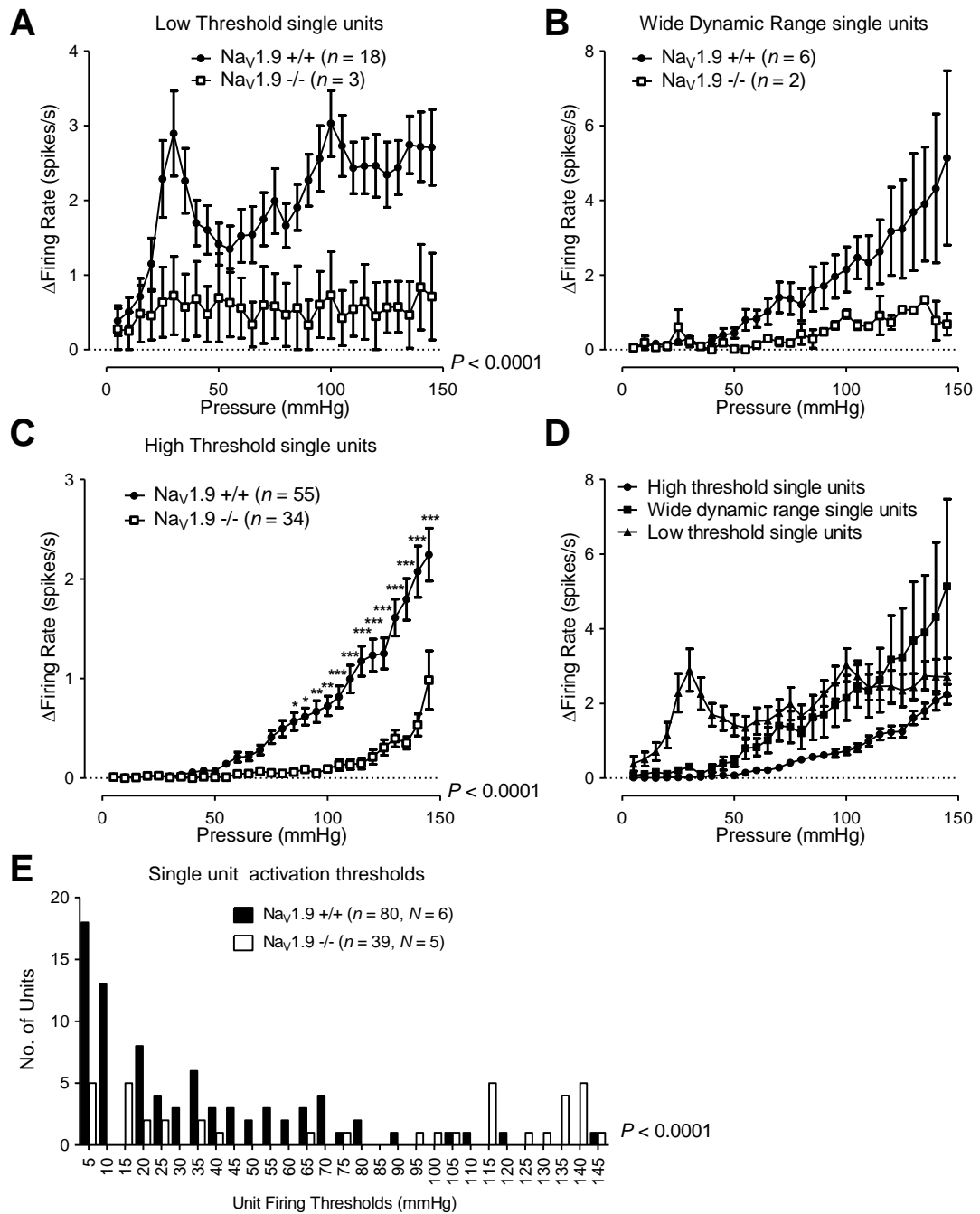


Figure 23. Post-hoc single unit analysis of multi-unit recordings made during 0-145mmHg ramp distensions. Response profiles to single units classified as low threshold (A), wide dynamic range (B) and high threshold (C) for both genotypes (2-way ANOVA with Bonferroni's post-hoc, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). D Mean response profiles for low threshold, wide dynamic range and high threshold units from $Na_V1.9 +/-$ mice presented on the same plot. The three different response profiles can clearly be observed. E Analysis of activation thresholds revealed a rightward shift and an increase in the proportion of units with activation thresholds of > 100 mmHg in $Na_V1.9 -/-$ mice ($p < 0.0001$, Chi-squared test).

3.4.2 POST-HOC SINGLE-UNIT ANALYSIS OF MULTI-UNIT RECORDINGS TO RAMP DISTENSION AND TEASED SINGLE-FIBRE RECORDINGS OF MECHANICAL STIMULI IN LUMBAR SPLANCHNIC NERVE

Deficits in afferent mechanosensation observed in ramp and phasic distension protocols could be due to increased adaptation above threshold and/or altered thresholds for activation. This was investigated by post-hoc single-unit analysis of multi-unit recordings during ramp distension and by teased-fibre recordings in a flat-sheet preparation. Using strict action potential sorting criteria, 80 single units were identified from 0 - 145mmHg ramp distensions in Nav1.9 ^{+/+} mice with an average activation threshold of 31.4 ± 3.5 mmHg and average firing rate of 1.3 ± 0.1 spikes/s. In the 39 single units identified during ramp distension in Nav1.9 ^{-/-} mice, there was a significant rightward shift to higher activation thresholds (73.1 ± 8.7 mmHg, $p < 0.001$) whilst the average firing rate per unit was reduced (0.6 ± 0.1 spikes/s, $p < 0.001$). Response profiles were compared between genotypes based on relative firing at high and low distending pressures and discriminated units were classified as low threshold (LT), wide dynamic range (WDR) or high threshold (HT; Figure 23). In Nav1.9 ^{-/-} preparations, response profiles of all three classifications were diminished with significant reductions observed in LT and HT units compared to Nav1.9 ^{+/+} animals ($p < 0.001$; Figure 23).

There are four subtypes of lumbar splanchnic nerve afferents characterised by their location and responses to stroke, blunt probe and circumferential stretch: mucosal, muscular and vascular; the latter of which can be further split into mesenteric and serosal afferents (Brierley et al., 2004). To identify which distension-sensitive afferent subtype was altered in Nav1.9 ^{-/-} mice, established

teased single-fibre recording techniques in a colonic flat-sheet preparation were used (see Figure 24).

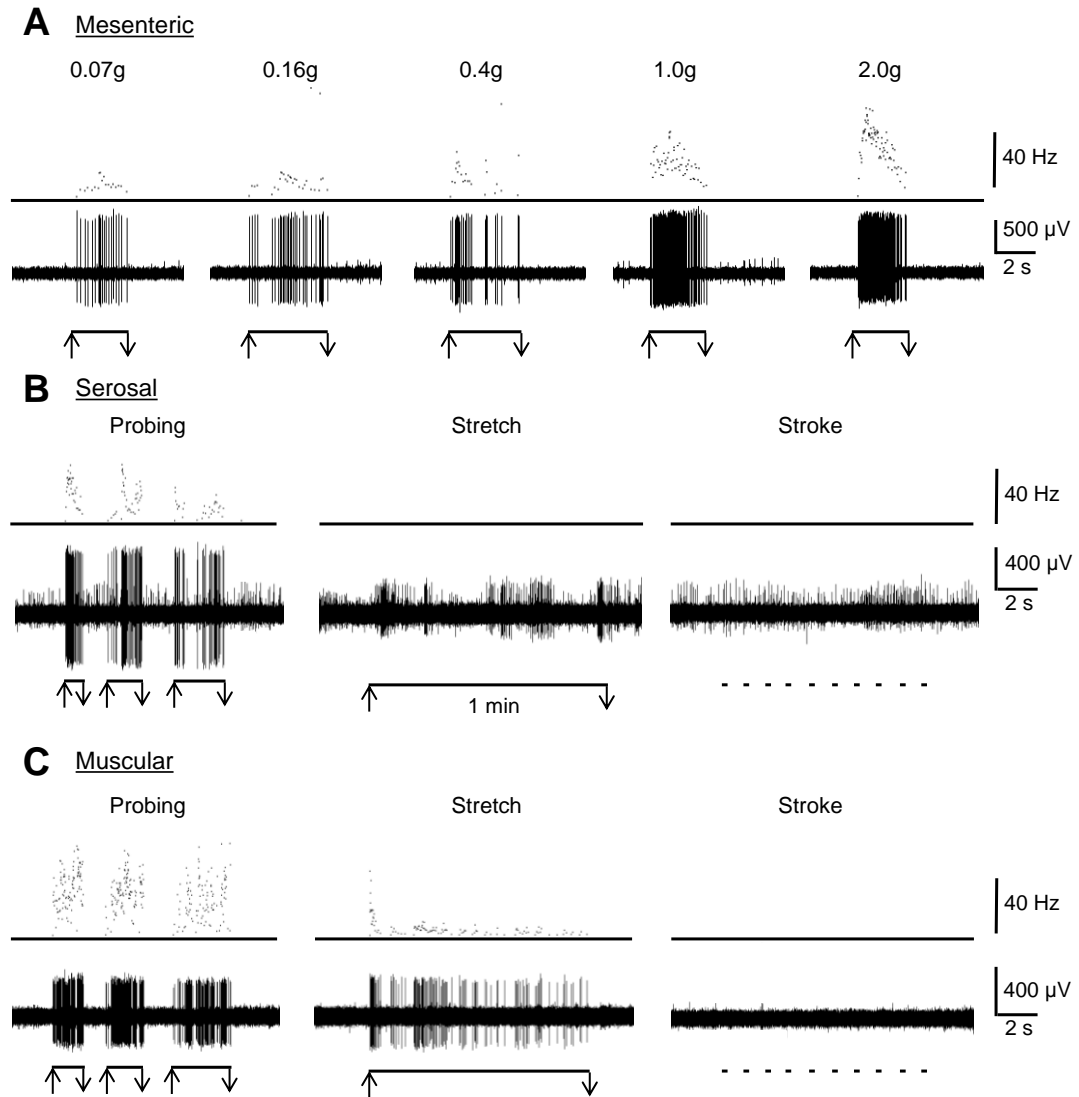


Figure 24. Example traces of mesenteric, serosal and muscular afferent LSN subtypes. A Von Frey hair mechanical stimulation of a vascular afferent fibre with receptive field in the mesenteric attachment. This fibre was unresponsive to circumferential stretch at 1g, 5g and 10g, and also to stroke of the mucosal surface by fine von Frey hair (10mg). B Example of vascular afferent with receptive field located intramurally within the colon wall and classified as serosal. Whilst not responsive to low-grade stretch (1g and 5g), a small proportion of serosal afferents could be activated by application of 10g stretch. C Example of muscular afferent with receptive field within the colon wall and responding to 5g and 10g stretch and von Frey hair probing.

Afferent fibres classified as mesenteric, serosal and muscular were consistently identified in both Nav1.9 +/+ and Nav1.9 -/- mice. Whilst initial efforts were taken to identify afferents sensitive to mucosal stroke none were identified. Given their scarcity in the splanchnic innervation of the gut ($\leq 4\%$) and likely limited involvement in nociception, their characterisation was not pursued (Brierley et al., 2004). As previously described, a small proportion (Nav1.9 +/+ : $\sim 13\%$ & Nav1.9 -/- : $\sim 21\%$) of serosal afferents also responded to greater (10g) circumferential stretch (Hughes et al., 2009). Stimulus-response curves to blunt probe with increasing weight of von Frey hairs of serosal and muscular units was unchanged between genotype ($p = 0.94$ and $p = 0.24$, respectively, see Figure 25A & C) with no significant differences observed in either mechanosensory thresholds or responses to circumferential stretch of the preparation (Figure 25D, F, G & H). A significant reduction in mesenteric afferent responses to von Frey hair probing, specifically at lower intensity probing (0.16-1g vFh); with a corresponding increase in mechanosensory threshold at these intensities observed in Nav1.9 -/- mice compared to wild-type animals (Figure 25B & E).

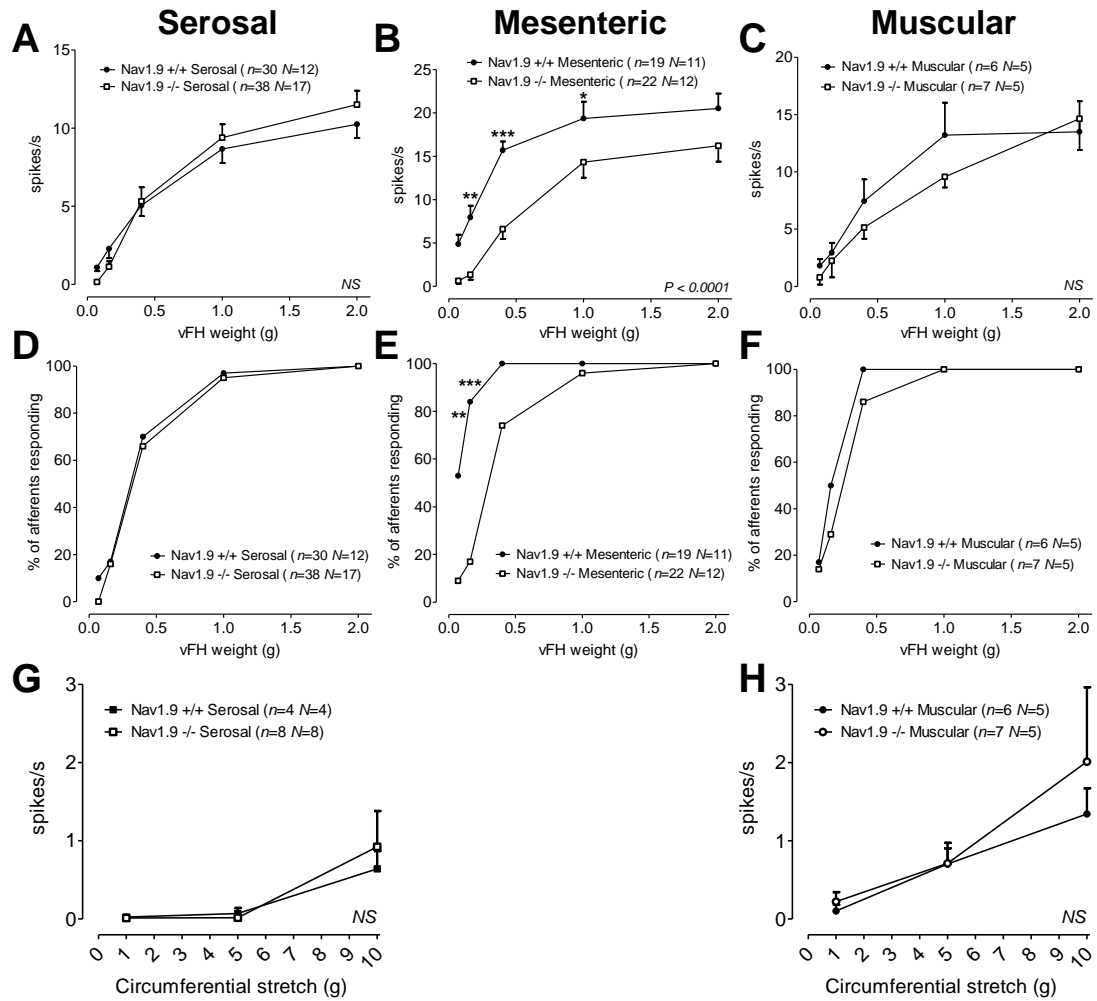


Figure 25. Stimulus-response curves to von Frey hair probing and circumferential stretch for afferent fibre subtypes in Nav1.9 +/- and Nav1.9 -/- mice. Stimulus-response curves to von Frey hair probing (0.07g – 2g) for serosal (A), mesenteric (B) and muscular (C) afferent fibres (2-way ANOVA with Bonferroni's post-hoc, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$). Associated activation thresholds of von Frey hair probing for serosal (D), mesenteric (E) and muscular (F) afferent fibres (Fisher's exact test at each probe weight, $**p < 0.01$; $***p < 0.001$). Responses to circumferential stretch (1g, 5g and 10g) in serosal and muscular afferent fibres (2-way ANOVA with Bonferroni's post-hoc).

Electrical stimulation of characterised single units in teased fibre recordings showed there was no difference in conduction velocity between control and Nav1.9 ^{-/-} mice (serosal, $p = 0.50$; mesenteric, $p = 0.90$; muscular, $p = 0.35$; Figure 27), signifying that action potential propagation is unaffected by loss of Nav1.9.

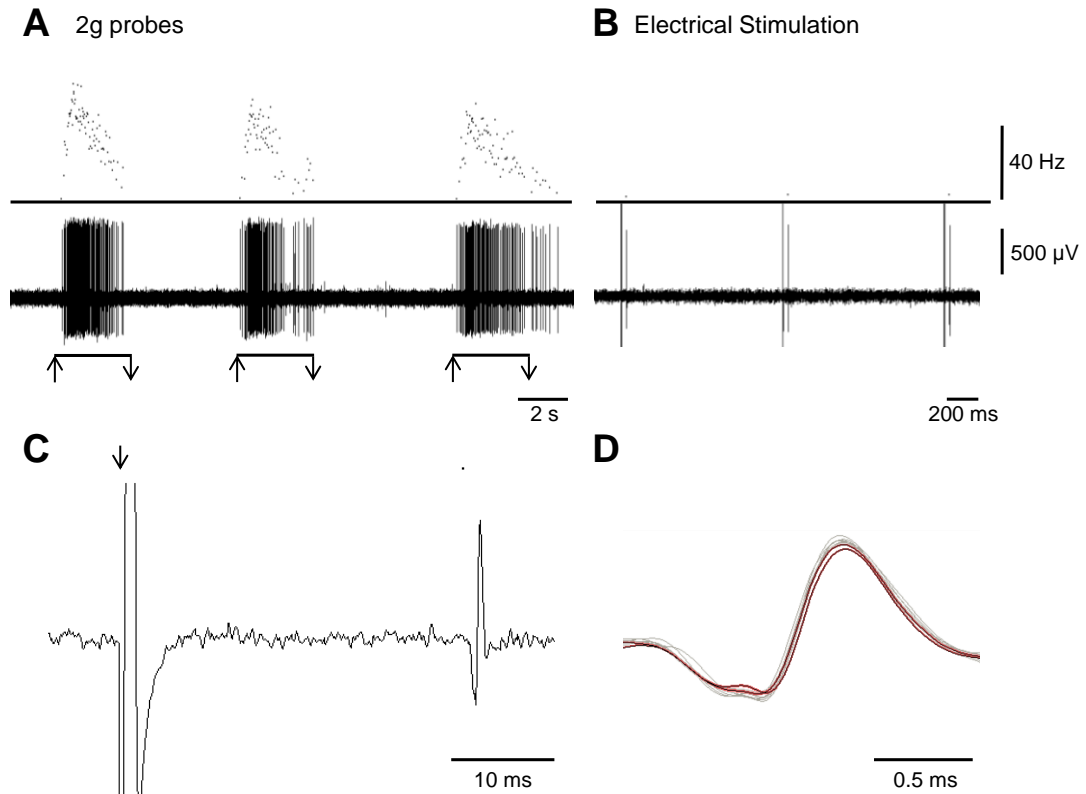


Figure 26. Conduction velocity determination by electrical stimulation. Conduction velocities were estimated by first identifying the afferent receptive field with von Frey hair probing (A). Receptive fields were then subjected to electrical stimulation (0.5Hz, 15V, 1ms) with concentric stimulating electrodes every second for 5 seconds (B; three stimulation artefacts with evoked action potential shown). If less than three action potentials were evoked by electrical stimulation, the electrode was repositioned and the receptive field re-stimulated. C To determine the conduction velocity, the time from stimulation artefact (arrow) to evoked action potential was measured (i.e. 33ms). The distance from receptive fields to recording electrode was then expressed as a fraction of the time taken for the action potential to travel this distance. In this example, this unit has a conduction velocity of 20mm/33ms or 0.61m/s. D Example waveform template matching. Five von Frey hair evoked action potentials (grey) overlaid with two electrically stimulated action potentials (red), confirming stimulation of the same unit.

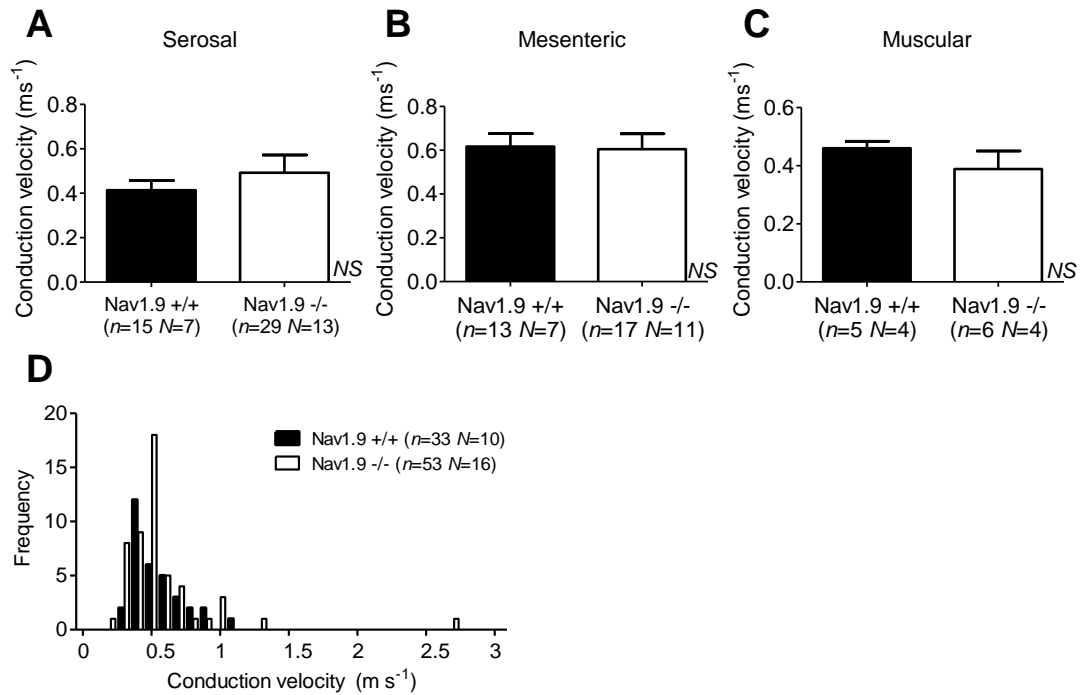


Figure 27. Conduction velocity of mechanosensitive afferent fibres in Nav_v1.9 +/+ and Nav_v1.9 -/- mice. Comparison of conduction velocities of electrically-evoked action potentials in serosal (A), mesenteric (B) and muscular (C) afferent fibre subtypes of Nav_v1.9 +/+ and Nav_v1.9 -/- mice (unpaired t-test). D Histogram of conduction velocity in 0.1 ms⁻¹ increments.

Together these data suggest that high-intensity mechanical stimulation of colonic afferents (e.g. 2g vFh probing, >95mmHg ramp distension and initial rapid phasic distension) is unaffected by Nav1.9 gene deletion. However knock-out of Nav1.9 appears to decrease the sensitivity and firing rates of mesenteric afferents to low-intensity stimulation, which likely accounts for the significant deficits observed during ramp distension and the post-hoc analysis of the associated single units. This is supported by the correlation between von Frey hair stimulation and distension of the intact bowel in mechanoreceptors located in the mesentery and suggests that this unit subtype comprises a significant proportion of the total afferent output during distension of the bowel (Brierley et al., 2008).

3.4.3 EFFECTS OF NAV1.9 DELETION ON RESPONSES OF COLONIC AFFERENTS TO INFLAMMATORY SOUP AND SUBSEQUENT MECHANICAL HYPERSENSITIVITY

The role for Nav1.9 in afferent responses to multiple inflammatory mediators was next investigated. To achieve this, single-fibre recordings were made in colonic flat sheet preparations, and receptive fields in the mesentery and serosa were identified. Discrete application of inflammatory soup (IS: bradykinin, ATP, histamine, serotonin and prostaglandin E₂) for 2 minutes in a small chamber surrounding the receptive field led to robust activation in 100% of serosal and 80% of mesenteric units in Nav1.9 +/+ mice (Figure 28). The increase in firing of single afferent fibres after IS was virtually abolished in Nav1.9 -/- mice, and only 43% of serosal ($p < 0.05$) and 56% of mesenteric ($p < 0.05$) units responded (Figure 28C).

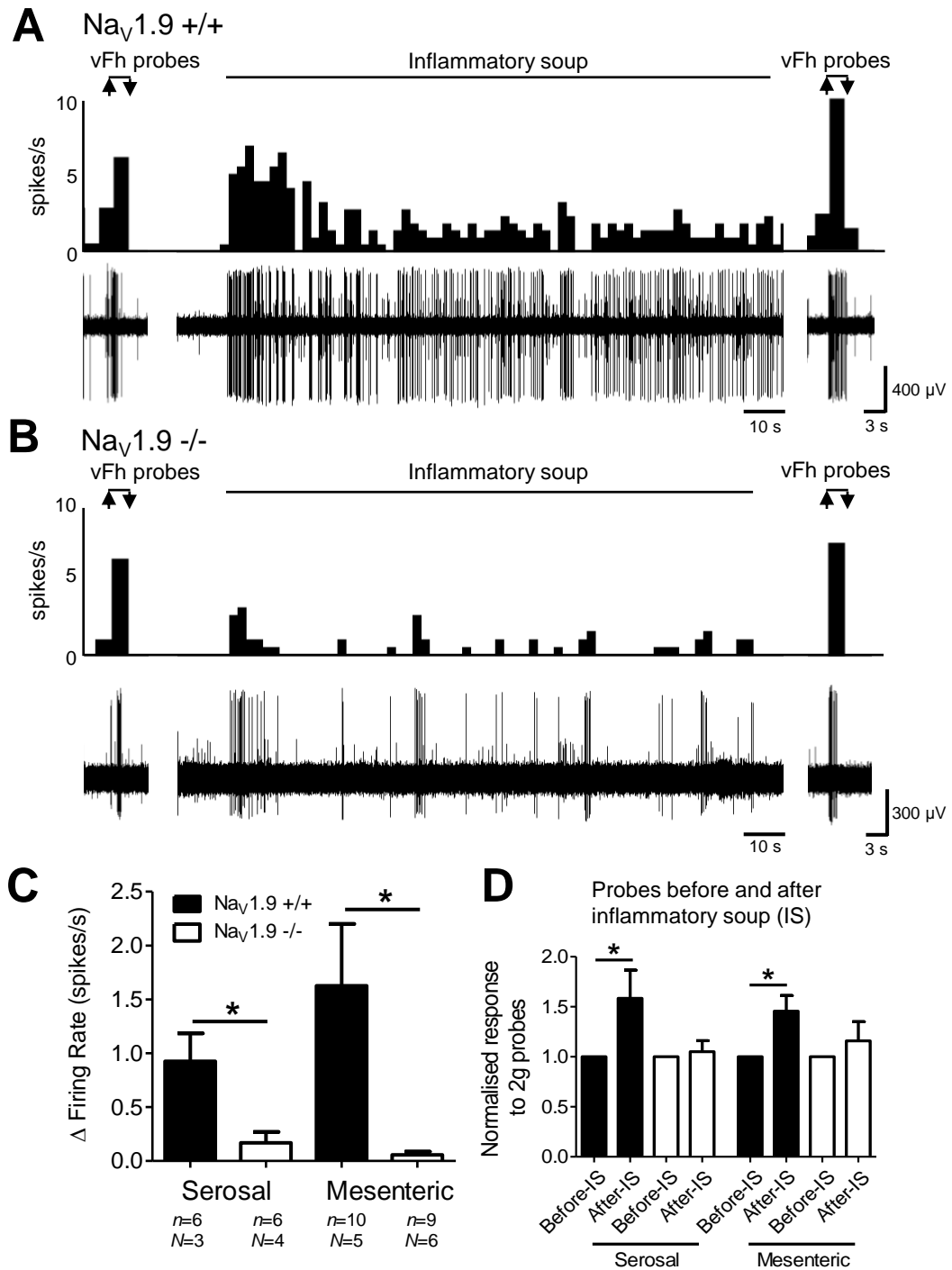


Figure 28. Chemical and mechanical stimulation of single fibre mechanoreceptive fields in mouse colon. Example colonic nerve activity to ring application (2 mins) of inflammatory soup (IS) over a receptive field located in the serosa of $\text{Na}_V1.9 +/+$ (A) and $\text{Na}_V1.9 -/-$ (B) mice. Probes with von Frey hair (2g) were performed prior to and after IS application. C Response to IS ring application in serosal and mesenteric receptive fields ($*p < 0.05$, unpaired t-test). D Afferent hypersensitivity post-IS application was observed in $\text{Na}_V1.9 +/+$ mice in both serosal and mesenteric units ($*p < 0.05$, paired t-test vs before-IS). This was not present in $\text{Na}_V1.9 -/-$ mice.

After responses to IS, mechanical hypersensitivity of colonic afferents was seen. Responses to 2g von Frey hair probing before and after IS application were compared in control animals and a significant increase in responses post-IS was observed ($p < 0.05$, Figure 28D). This is consistent with previous studies using the application of bradykinin (Brierley, Jones, et al., 2005). IS-induced mechanical hypersensitivity to a 2g probe was completely lost in Nav1.9^{-/-} mice (serosal, $p = 0.76$; mesenteric, $p = 0.63$, paired t-test), indicating that Nav1.9 may directly influence the mechanical hypersensitivity of visceral afferents induced by IS. This suggests that the mechanical hypersensitivity observed to intraluminal perfusion of inflammatory soup during 0-80mmHg ramp distension can be attributed to sensitisation of both serosal and mesenteric afferent subtypes.

3.4.4 Nav1.9 DELETION REDUCES COLONIC AFFERENT RESPONSES TO HUMAN TISSUE-DERIVED INFLAMMATORY SUPERNATANTS

Next, it was investigated whether ablation of Nav1.9 would alter responses not only to artificially reconstituted IS, but also to supernatants derived from inflamed human tissue. To do this, resected bowel tissue from patients undergoing surgery for inflammatory bowel disease (IBD: Crohn's disease (CD) and ulcerative colitis (UC)) was incubated in Krebs (see Methods) to generate individual IBD supernatants. Separately, macroscopically normal tissue taken from bowel cancer resections (> 10cm away from the cancer itself, cancer margins and lymph nodes) was used to generate control supernatants. Such control supernatants enabled possible excitatory effects caused by surgical manipulation, removal of tissue and ischemia to be discounted from afferent

activation driven by inflammatory mediators released from native tissue. Importantly, no further steps were taken to concentrate the tissue supernatant in any way. To confirm the inflammatory status of the supernatants, cytokine quantification for IL-6, IL-8, IL-1 β , GM-CSF and TNF- α was performed. In control supernatants, there were detectable levels of IL-6 (7.5 ± 3.3 pg/ml) and IL-8 (43.4 ± 13.8 pg/ml); with levels of the remaining cytokines below detection limits (Figure 29D). Cytokine levels were all significantly elevated in IBD supernatants (IL-1 β : 35.4 ± 22.7 ; IL-6: 598.6 ± 276.3 ; GM-CSF: 37.5 ± 14.9 ; TNF- α : 31.5 ± 23.6 ; IL-8: 3419.3 ± 2083.7 pg/ml; each cytokine vs. control, $p < 0.05$), with cytokine levels greatest in supernatants generated from Crohn's disease tissue compared to that of ulcerative colitis. It should be mentioned that the influence of immunosuppressant and anti-TNF α antibody therapies on cytokine production was not accounted for during patient selection. Supernatants were initially applied to mouse whole-nerve lumbar splanchnic recordings in a cannulated tubular preparation. IBD supernatants elicited a robust increase in afferent firing compared to control supernatants in wild-type animals (3.5 ± 0.5 spikes/s vs. control supernatant 0.7 ± 0.1 spikes/s, $p < 0.01$, Figure 29C). In Nav1.9 $-/-$ mice, the response to IBD supernatants was greatly attenuated and almost comparable in magnitude to control supernatant response in wild-type mice (1.2 ± 0.3 spikes/s, vs. Nav1.9 $+/+$ mice, $p < 0.01$).

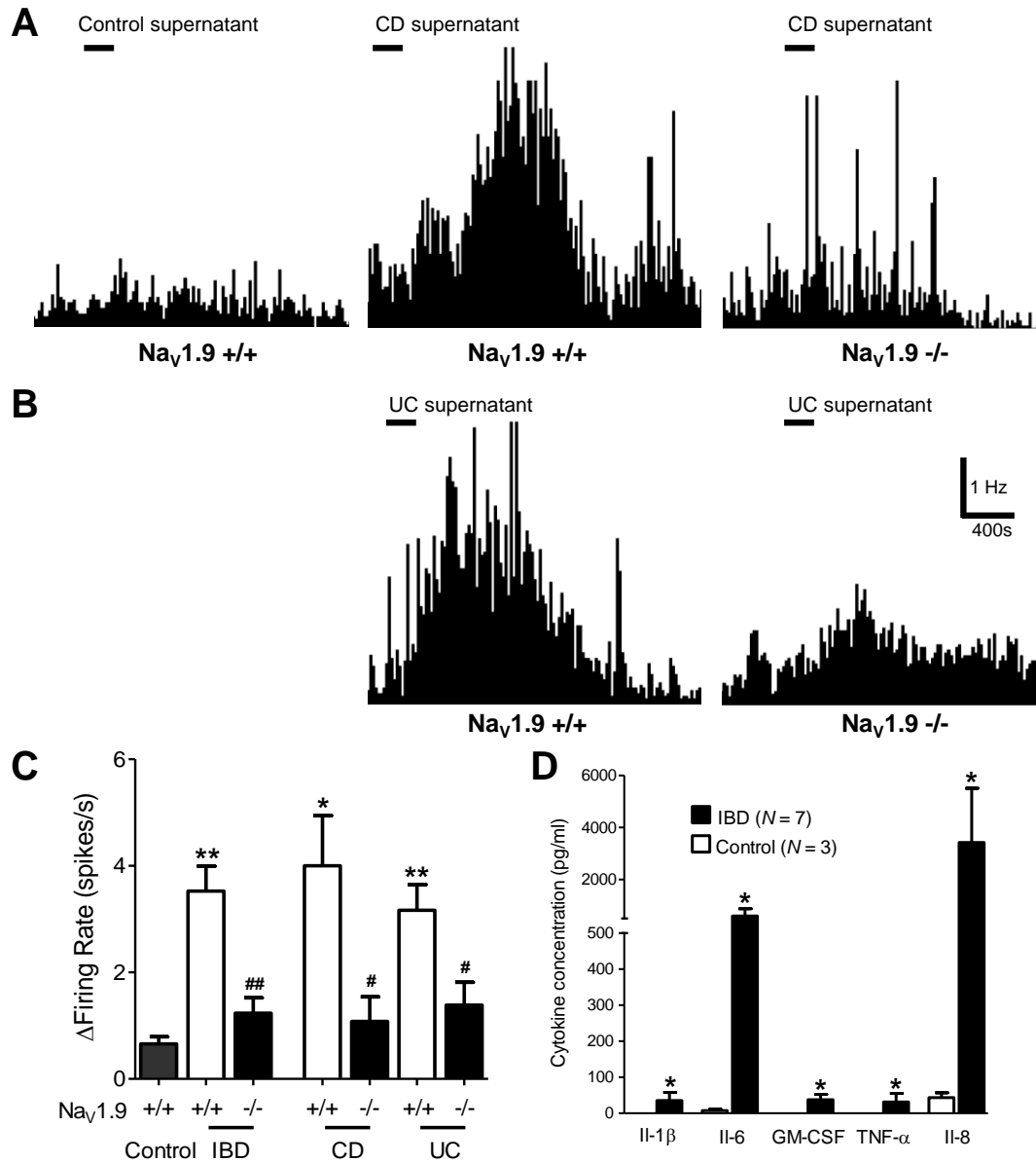


Figure 29. The effect of inflammatory bowel disease (IBD) supernatants on colonic nerve activity. A Example rate histograms of colonic nerve activity to control supernatant in Na_V1.9+/+ mice and to Crohn's disease (CD) tissue supernatant in Na_V1.9+/+ and Na_V1.9-/- mice. B Example rate histograms of colonic nerve activity to ulcerative colitis (UC) supernatant in both Na_V1.9+/+ and Na_V1.9-/- mice. C Peak change in activity following addition of control (*N* = 3), IBD supernatants in Na_V1.9+/+ (*N* = 7) and Na_V1.9-/- mice (*N* = 6). In addition, CD and UC responses are plotted separately to the right of the panel (*N* = 3-4 per genotype). **p* < 0.05, ***p* < 0.01 vs control; #*p* < 0.05, ##*p* < 0.01 Na_V1.9+/+ vs Na_V1.9-/. D Cytokine (IL-1β, IL-6, GM-CSF, TNF-α & IL-8) levels in IBD (*N* = 7) and control (*N* = 3) supernatants applied to afferent nerve recordings. **p* < 0.05 vs control.

After confirming that human tissue-derived inflammatory supernatants could indeed excite colonic afferents, the effect of CD supernatants was investigated by direct application in a small chamber over isolated receptive fields during single-fibre recordings in a flat sheet preparation. Crohn's disease supernatants were chosen over ulcerative colitis supernatants due to the comparatively larger afferent excitation evoked during whole-nerve tubular experiments and also due to the greater incidence of abdominal pain in Crohn's disease vs. ulcerative colitis patients (see Figure 29). The responses to ring application of CD supernatant were less robust and frequent in comparison to experimental IS in wild-type animals, with 64% of serosal (1.37 ± 0.36 spikes/20s) and 50% of mesenteric afferents (1.13 ± 0.22 spikes/20s) responding (Figure 30D). The proportions of units responding in Nav1.9 $-/-$ mice were comparable ($p = 1.00$, Fisher's exact test, Figure 30D), however the degree of afferent activation elicited by ring application of CD supernatants was significantly less (serosal: 0.31 ± 0.11 spikes/20s, $p < 0.05$ vs Nav1.9 $+/+$; mesenteric: 0.42 ± 0.16 spikes/20s, $p < 0.05$ vs Nav1.9 $+/+$). No mechanical hypersensitivity was observed after CD supernatant in either Nav1.9 $+/+$ or Nav1.9 $-/-$ mice to 2g von Frey hair probe (Figure 30E & F), regardless of whether or not afferents showed direct excitation to the supernatant. It is clear that Nav1.9 contributes to visceral afferent excitation by both experimental and natural inflammatory milieu of differing composition.

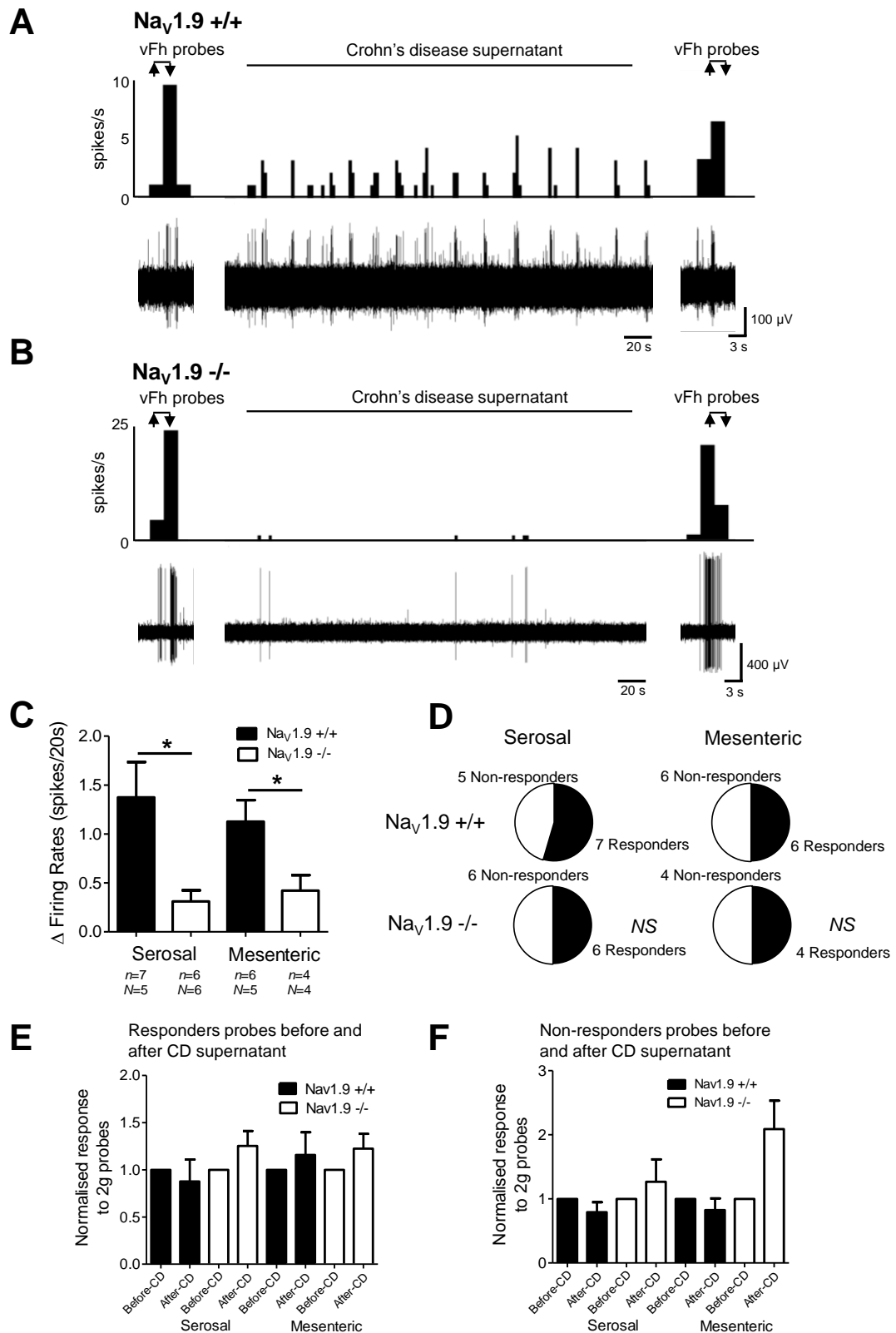


Figure 30. Mechanical sensitivity and stimulation of single fibre mechanoreceptive fields in mouse colon by human inflammatory supernatant. Example colonic nerve activity to ring application (5 mins) of Crohn's disease inflammatory supernatant over a receptive field located in the serosa of Nav_v1.9 +/+ (A) and Nav_v1.9 -/- (B) mice.

Probes with von Frey hair (2g) were performed prior to and after CD supernatant application. C Response to CD supernatant ring application in serosal and mesenteric receptive fields ($*p < 0.05$, unpaired t-test). D Number of responsive versus non-responsive serosal and mesenteric units when CD supernatants were applied (Fisher's exact test). E & F Afferent hypersensitivity post-IS application was not observed in Na_v1.9 +/+ mice in either serosal or mesenteric units, independent of whether or not afferents showed direct excitation to CD supernatant (paired t-test vs before-supernatant).

3.5 SUMMARY OF KEY FINDINGS

3.5.1 COLONIC AFFERENT EXCITATION TO MECHANICAL STIMULI IN Nav1.9 -/- MICE

1. In a tubular preparation, rapid phasic distension to 80mmHg evoked equivalent colonic afferent excitation in both Nav1.9 +/+ and Nav1.9 -/- mice.
2. Repeat phasic distension led to adaptation in the response in Nav1.9 +/+ mice, with responses stabilising by the 4-6 repeat distension.
3. The degree of run-down of responses to repeat distension was significantly greater in Nav1.9 -/- mice.
4. During slow ramp distension to 80mmHg, Nav1.9 +/+ mice produced a linear stimulus-response, with responses in Nav1.9 -/- mice almost completely abolished.
5. Supra-physiological ramp distension to 145mmHg led to increased afferent firing in Nav1.9 +/+ mice. Responses in Nav1.9 -/- mice remained very low at distension pressures less than 95mmHg. Above this pressure, an exponential increase in Nav1.9 -/- afferent firing was observed, with responses almost reaching that of Nav1.9 +/+ mice at 145mmHg.
6. Intraluminal perfusion of inflammatory soup (IS) led to mechanical sensitisation of responses to ramp distension to 80mmHg in Nav1.9 +/+ mice. This was also true of responses from Nav1.9 -/- mice, but to a lesser extent and not at pressures below 50mmHg.

7. Post-hoc analysis of activation thresholds of single units from ramp distensions to 145mmHg in Nav1.9 -/- mice were significantly increased compared to Nav1.9 +/+ mice, and firing rates were significantly decreased.
8. In teased fibre recordings from flat-sheet preparations, afferent fibres characterised as serosal and muscular had comparable stimuli-response curves to mechanical stimuli by von Frey hairs and circumferential stretch, irrespective of genotype. Activation thresholds were also unchanged in these subtypes.
9. However, mesenteric afferents from Nav1.9 -/- mice exhibited reduced von Frey hair stimulus-response curves, and a corresponding rightward shift in activation thresholds.

3.5.2 CONDUCTION VELOCITIES OF COLONIC AFFERENTS FROM Nav1.9 -/- MICE

1. In a flat-sheet preparation, teased fibre recordings of serosal, mesenteric and muscular afferents showed no difference in conduction velocities between genotypes.

3.5.3 EFFECT OF INFLAMMATORY SOUP ON THE EXCITATION AND MECHANICAL HYPERSENSITIVITY OF COLONIC AFFERENTS IN Nav1.9 -/- MICE

1. Application of inflammatory soup to discrete serosal and mesenteric receptive fields in a flat-sheet preparation evoked robust afferent discharge in Nav1.9 +/+, but not Nav1.9 -/-, mice.
2. Post-inflammatory soup mechanical hypersensitivity to 2g von Frey hair probe was observed in Nav1.9 +/+ mice. This was completely lost in Nav1.9 -/- mice.

3.5.4 EFFECT OF HUMAN INFLAMMATORY SUPERNATANTS ON EXCITATION AND MECHANICAL HYPERSENSITIVITY OF COLONIC AFFERENTS IN Nav1.9 -/- MICE

1. In a tubular preparation, bath superfusion of both Crohn's disease and ulcerative colitis supernatants, but not control supernatants, evoked robust activation of Nav1.9 +/+ colonic afferents, which was lost in Nav1.9 -/- mice.
2. Application of Crohn's disease supernatants to discrete receptive fields in the serosa and mesentery in a flat-sheet preparation from Nav1.9 +/+ mice led to modest afferent discharge in approximately 50% of fibres. This response was significantly reduced in Nav1.9 -/- mice, although equivalent numbers of fibres responded.
3. No post-Crohn's disease supernatant mechanical hypersensitivity to 2g von Frey hair probe was observed in either Nav1.9 +/+ or Nav1.9 -/- mice.

3.6 DISCUSSION

The data above shows that Nav1.9 is required for the direct excitatory responses of nociceptive colonic afferents to inflammatory mediators from diseased tissue, and for mechanical sensitisation by these same mediators. Nav1.9 is also involved in determining the mechanosensory thresholds of a subset of colonic afferents with receptive fields in the mesenteric attachments, and thereby determining responses to rapid and/or intense distension in the intact colon. Nav1.9 may also play a role in the persistence of responses to repeated noxious distension, which would otherwise attenuate over time.

Given the polymodal nature of visceral nociceptors, there are likely points of convergence in the molecular signalling pathways underlying action potential generation at their endings (Su & Gebhart, 1998). These will provide valuable targets for future treatments, which could counteract the mechanical hypersensitivity induced by ongoing inflammation. Voltage-gated sodium channels may represent such a convergence point in this signalling cascade. Of the peripherally expressed sodium channels, valuable insight has been obtained regarding the function of Nav1.7 and Nav1.8 in visceral chronic pain, not least due to causal links to human pain syndromes (Fertleman et al., 2006; Reeder et al., 2013). However, the functional evidence for a role of Nav1.9 in visceral afferents has not been extensively explored before, despite its appealing biophysical characteristics. The data presented here suggests that Nav1.9 represents an important regulator of specific aspects of sustained visceral afferent excitability, especially in response to inflammatory mediators.

3.6.1 AFFERENT CHEMOSENSITIVITY IN *Nav1.9* ^{-/-} MICE

The persistent sodium current defined by *Nav1.9* can be greatly enhanced by inflammatory mediators acting at GPCRs via $G_{q/11}$ /PKC and $G_{i/o}$ /PKA intracellular signalling pathways (Baker, 2005; X. Chen et al., 2011; Maingret et al., 2008; Rush & Waxman, 2004), which can result in neuronal depolarisation and increased afferent excitability. Mediators applied individually to dissociated DRG neurones may not be sufficient to evoke such *Nav1.9* current enhancements, with Maingret *et al.* suggesting that multiple inflammatory mediators are required (Maingret et al., 2008); whilst others have shown that ATP or PGE₂ applied alone can increase *Nav1.9* current amplitudes (Baker, 2005; Rush & Waxman, 2004). The data presented here extends these observations by examining the contribution of *Nav1.9* to nociceptor activation at the level of the nerve ending. Responses of individual receptive fields in the mesentery and serosa to the application of experimental inflammatory soup (consisting of bradykinin, histamine, PGE₂, ATP and 5HT) were significantly attenuated in *Nav1.9* ^{-/-} mice (see Figure 28). Basal whole-nerve firing rates were also significantly reduced following intraluminal perfusion of IS, demonstrating that *Nav1.9* is required in the activation of nerve fibres by these mediators (Figure 21). To explore this further we generated supernatants by incubating resected tissue from patients with IBD. These disease derived IBD supernatants caused increases in afferent activity in whole nerve recordings from mouse colon, and in single unit recordings from serosal and mesenteric afferents; both of which were significantly attenuated in *Nav1.9* ^{-/-} mice (see Figure 29). The degree of nerve excitation observed following application of IBD

supernatants was comparable to those previously reported following application of irritable bowel syndrome supernatants to mesenteric nerve recordings of rat (Barbara et al., 2007). The attenuation of responses to human IBD supernatants in Nav1.9 $-/-$ mice supports a more general role for Nav1.9 in afferent excitability by multiple inflammatory pathways potentially including those evoked by disease-relevant cytokines. In whole tissue preparations such as those employed in the current study, it is highly unlikely that visceral afferent terminals are solely exposed to just IS or IBD supernatant, with these experimental mediators likely to act alongside the endogenous inflammatory milieu released by adjacent tissues. Given the exposure to multiple mediators, it is likely that Nav1.9 current potentiation is occurring in these afferents, however these findings do not restrict an unenhanced Nav1.9 current from exhibiting significant effects on resting membrane potential, neuronal excitability and transduction at visceral afferent terminals. Whilst constituents of the inflammatory soup, including ATP and PGE₂, can enhance currents generated by other sodium (e.g. Nav1.8 (Baker, 2005)) or ion channels (Schicker, Chandaka, Geier, Kubista, & Boehm, 2010; Tominaga, Wada, & Masu, 2001), this data demonstrates that visceral afferent excitability to these mediators appears highly dependent on Nav1.9. Collectively, this suggests that during human inflammatory disease where multiple inflammatory stimuli are present, Nav1.9 contributes significantly to visceral afferent activation.

3.6.2 EFFECT OF $Na_v1.9$ DELETION ON MECHANICAL HYPERSENSITIVITY TO INFLAMMATORY STIMULI

Deletion of $Na_v1.9$ also caused significant deficits in the development of mechanical hypersensitivity following application of inflammatory mediators. These deficits were investigated using different mechanical stimuli, including von Frey probing of isolated visceral nociceptor receptive fields and ramp distension of the colon into noxious pressure range. The response to noxious ramp distension was sensitised by intraluminal perfusion of inflammatory soup, which, at least during lower distension pressures was dependent on $Na_v1.9$ (Figure 21). Post-IS mechanical hypersensitivity to von Frey hair probing was also observed in both serosal and mesenteric afferent fibre subtypes in wild-type mice. This was comparable to previously reported mechanical hypersensitivity in these afferent subtypes following application of bradykinin (Brierley, Jones, et al., 2005). This mechanical hypersensitivity was completely lost in $Na_v1.9$ $-/-$ animals and suggests that $Na_v1.9$ has a central role in the sensitisation of afferent fibres to mechanical stimuli. Whilst application of Crohn's disease (CD) supernatants did evoke moderate excitation of some mesenteric and serosal afferents, CD supernatants did not induce a mechanical hypersensitivity to von Frey hair probe in either $Na_v1.9$ $+/+$ or $Na_v1.9$ $-/-$ mice. It is possible that this is due to the combination of inflammatory mediators in IBD pathology being unable to evoke mechanical hypersensitivity in these afferent fibres, but may also be a result of the relatively weak excitatory stimulus provided by these supernatants compared to responses following the application of experimental IS. There is certainly a subset of IBD patients that are normosensitive or hyposensitive to colorectal distension (Bernstein et al.,

1996; Chang et al., 2000). Indeed, faecal supernatants from ulcerative colitis patients induce hyposensitivity in mice during VMR to CRD (Annaházi et al., 2009). As such, no change, or even a reduction, in response to von Frey hair probe post-CD application may have been predicted in these single-fibre studies (see Figure 30). By contrast, IBS supernatants have been shown to robustly evoke mechanical hypersensitivity in animal models (Annaházi et al., 2009; Cenac et al., 2007). Visceral hypersensitivity is also observed in a subset of IBD patients presenting with IBS, in so called IBD-IBS (Grover et al., 2009). It would be interesting to examine IBS-supernatant-induced mechanical hypersensitivity in Nav1.9 $-/-$ mice and determine whether these are more analogous to changes induced by experimental IS. Collectively, these data do suggest that experimental IS and IBD supernatants evoke their actions through differing inflammatory pathways. In both cases, this is consistent with the proposed role for Nav1.9 in regulating afferent nerve terminal sensitisation during inflammation.

Further experiments investigating the effect of Nav1.9 deletion on long-term chronic inflammation are warranted to fully understand the ability of Nav1.9 to modulate afferent excitability.

3.6.3 MECHANOSENSATION OF COLONIC AFFERENTS IN NAV1.9 $-/-$ MICE

The results also implicate Nav1.9 in the mechanosensation of colonic afferents. These were investigated using repeated phasic and ramp distension of the colon into a noxious pressure range and by von Frey hair probing of isolated visceral receptive fields. Such studies have proven invaluable in the development of our current understanding of how noxious mechanical stimulation of the gut is

transduced by visceral nociceptors (Booth et al., 2008; Brierley et al., 2008; Feng, La, Schwartz, et al., 2012). Von Frey hair probes were used to elicit brief, intense and graded mechanical stimuli. There are populations of colonic mechanoreceptors with receptive terminals on blood vessels in both the mesentery and intramurally within the colon, which respond to high-threshold mechanical stimulation and are believed to comprise a major type of nociceptor (Brookes et al., 2013). Importantly, these same primary afferents also respond to intraluminal distension at pressures that evoke pain in other species *in vivo* (Schmulson, Chang, Naliboff, Lee, & Mayer, 2000; Whitehead et al., 1990). Mechanical activation of afferents with receptive fields located in the serosa is unaffected by Nav1.9 gene deletion (Figure 25). This is also true of mechanoreceptors characterised as muscular, which respond to low intensity circumferential stretch (5g) and vFh probing identically, regardless of genotype. However, mechanosensory responses of afferents with receptive fields in the mesentery are significantly attenuated, with a consistent increase in activation thresholds observed in Nav1.9 -/- mice. As mentioned previously, whilst serosal and mesenteric sub-populations are thought to represent a common class of “vascular” visceral afferent fibre (Brookes et al., 2013), these two classes are differently altered not just in Nav1.9 knock-outs, but also in ASIC2 -/- mice (Page, Brierley, et al., 2005). Furthermore, serosal and mesenteric afferents have different wild-type sensitivities to distension and von Frey hairs (Song et al., 2009). Whether differences in the expression of mechanotransducers, or other known regulators of neuronal excitability (such as T-type calcium channels or HCN2), present in serosal versus mesenteric afferents contributes to the importance of Nav1.9 in regulating afferent firing remains to be seen

(Emery et al., 2011; Sekiguchi & Kawabata, 2013). As such, Nav1.9 appears to regulate mesenteric afferent excitability to mechanical stimuli to a greater extent than when compared to afferents with intramural receptive fields.

The data shows that excitation of visceral afferents by initial rapid phasic distension is unaffected by Nav1.9 deletion. This observation supports the data generated using high intensity vFh (2g) stimulation, which produced comparable responses across all types of units regardless of genotype. It suggests that the reduction in afferent excitability caused by the loss of Nav1.9 may be overcome by greater mechanical stimulation, and presumably greater activation of stimulus transducing channels. In contrast to the initial phasic distension, Nav1.9 is required for the maintenance of afferent sensitivity to persistent mechanical stimuli. The desensitisation of responses typically seen to repeat colorectal distension (Kamp et al., 2003; Sivarao et al., 2007) did not stabilise in Nav1.9 ^{-/-} mice, but instead continued to decline with subsequent distensions (Figure 20). One possible explanation for this observation, that is worthy of further investigation, is that repeated mechanical stimuli may progressively desensitise activity in mechanosensitive channels, such as TRPV4, TRPA1, ASIC3, however a concurrent up-regulation of Nav1.9 maintains a consistent level of afferent activation. This mechanism could be crucial *in vivo*, where pain is evoked by repeated contractions of the colon around a bolus or stricture, and is amplified in IBS (Brookes et al., 2013). Further, this may be relevant in relation to afferent adaptation during experimental models of visceral pain, such as the visceromotor response (VMR) to colorectal distension. Typically, a set of distensions (e.g. 4-8) is applied to achieve a stable response,

at which point the intervention is applied (Kamp et al., 2003; Sivarao et al., 2007). Therefore the relative importance of mechanosensory pathways in an afferent having undergone stimuli adaptation may differ from those present in a naïve state. Consistent with this proposed role for Nav1.9 in persistent or sustained stimuli, a substantial reduction in the afferent response to ramp colorectal distension (0-80mmHg) in Nav1.9^{-/-} tissue was observed at all pressures including those within the noxious range. When these pressures were extended into the supra-maximal range (100-150mmHg), increases in afferent activity were observed in both genotypes. This is in keeping with the previous hypothesis that increased stimulus strength may overcome the loss of excitability following deletion of Nav1.9. Post-hoc single unit analysis of responses to ramp distension showed that units characterised as low-threshold, wide dynamic range and high-threshold, demonstrated reduced firing rates. This is consistent with the observed changes in activation threshold and reduced firing rates of mesenteric afferent fibres to von Frey hair probe in a flat-sheet preparation, and suggests that mesenteric afferent response contributes significantly to activity evoked by fluid ramp distension at varying pressures. It is perhaps important to note that the distension paradigms used, rapid phasic vs. slow ramp, model markedly different physiological stimuli and will activate a range of afferent subtypes. This is relevant given the preferential activation of splanchnic afferents by phasic distension over ramp distension in man (Lembo et al., 1994).

Finally, no difference was observed in conduction velocities of visceral afferent fibres between genotypes, suggesting that action potential propagation is not

affected by Nav1.9 ablation. The conduction velocities of afferents recorded were in the C-fibre range, consistent with previous studies (Bielefeldt & Davis, 2008; Brierley et al., 2008; Cervero & Sharkey, 1988; Malin et al., 2009).

3.6.4 ISOLATION OF EXTRINSIC AFFERENT ACTIVITY FROM ENTERIC NERVOUS SYSTEM

Considerable efforts were taken to ensure that changes observed in afferent activation in this study were not confounded by the loss of Nav1.9 within intrinsic primary afferent neurones (IPANs) of the enteric nervous system (ENS), which are known to express Nav1.9 (Padilla et al., 2007; Rugiero et al., 2003). Although the contribution of second order enteric neurones projecting out of the gut (intestino-fugal fibres) cannot be ruled out in the recordings presented here, at the recording site central to the inferior mesenteric ganglia, these would proportionally represent a small minority of the total splanchnic outflow (Luckensmeyer & Keast, 1995). It is also currently unknown whether intestino-fugal fibres express Nav1.9. The potential impact of the loss of Nav1.9 from IPANs on local reflexes was controlled by the inhibition of intrinsic smooth muscle tone by inclusion of both nifedipine and atropine to the perfusion buffer. Finally cross-talk between the ENS and extrinsic afferents innervating the colon does not mediate extrinsic afferent sensitivity (Mueller et al., 2009). Indeed, in the aganglionic rectum of piebald-lethal mice, extrinsic mechanoreceptor development and function is largely unaffected by complete loss of enteric ganglia (Spencer, Kerrin, Zagorodnyuk, et al., 2008). The methodological steps outlined above, in conjunction with both the magnitude of the effects observed and the paucity of evidence for cross-talk between the ENS and extrinsic

afferents mediating extrinsic afferent sensitivity, would strongly suggest that the changes observed are due to modulation of neuronal excitability by Nav1.9 in visceral extrinsic afferents.

3.6.5 CONCLUSIONS

In conclusion, these data show that Nav1.9 represents a significant mechanism responsible for regulating visceral nociceptor sensitivity to mechanical stimuli, and to sensitising inflammatory mediators, including those released by human inflammatory bowel disease. The data further supports a role for Nav1.9 in the regulation of visceral afferent excitability and sensitivity in nociception (see Figure 31).

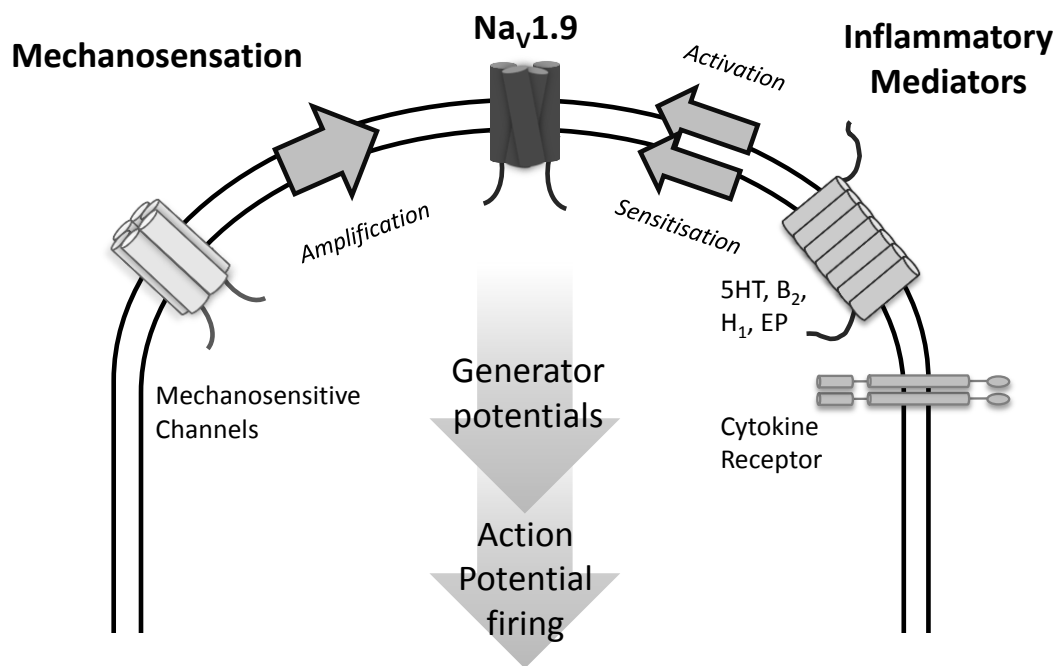


Figure 31. Schematic illustration of Na_V1.9 interacting with membrane-bound receptors and ion channels in a visceral afferent terminal. When present at the visceral afferent terminal, Na_V1.9, as well as contributing to setting the resting membrane potential acts to amplify generator potentials evoked by mechanosensitive channels, and functions as a key transducer of inflammatory mediators and other sensitising stimuli.

CHAPTER 4: PURINERGIC SIGNALLING AND $\text{Na}_v1.9$ IN GUT VISCERAL AFFERENT FIBRES

4.1 INTRODUCTION

Inflammatory bowel disease is characterised by chronic and relapsing diarrhoea, fatigue and bleeding accompanied by abdominal pain, which can continue during states of disease remission (Bielefeldt et al., 2009). One endogenous algogenic mediator of bowel discomfort and pain is adenosine 5'-triphosphate (ATP), which is released from the mucosal epithelium during distension (Burnstock, 1999) and is released in greater amounts during colitis (Calvert et al., 2008; Shinoda et al., 2009; Wynn et al., 2004). Both P2X and P2Y receptors may be activated by extracellular ATP, suggesting that they both contribute to altered sensation in IBD. Of the reported seven P2X subtypes (P2X₁₋₇), P2X₂ and P2X₃ receptor subunits are widely expressed in peripheral neurones and are thought to contribute significantly to the transduction of nociceptive visceral signals in the bowel (Burnstock, 1999). The eight members of the P2Y receptor family (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁₋₁₄) respond to endogenous purinergic nucleotides (ATP, ADP, UTP and UDP) released from various tissues into the extracellular environment (Gerevich & Illes, 2004)(see Figure 32). Activation of P2Y receptors in sensory neurones is capable of evoking action potential firing (Yousuf, Klinger, Schicker, & Boehm, 2011), inducing Ca²⁺ flux (Borvendeg, Gerevich, Gillen, & Illes, 2003; Ceruti, Fumagalli, Villa, Verderio, & Abbracchio, 2008) and activating intracellular phosphorylative cascades (Molliver, Cook, Carlsten, Wright, & McCleskey, 2002). In addition, P2Y receptors may activate cutaneous afferent fibres and regulate mechanosensitivity (Stucky, Medler, & Molliver, 2004; Tominaga et al., 2001). As such, P2Y receptor signalling is likely to contribute to

mechanosensation and nociception under both physiological and pathophysiological conditions. The function and known expression of individual purinoceptors is outlined in detail below.

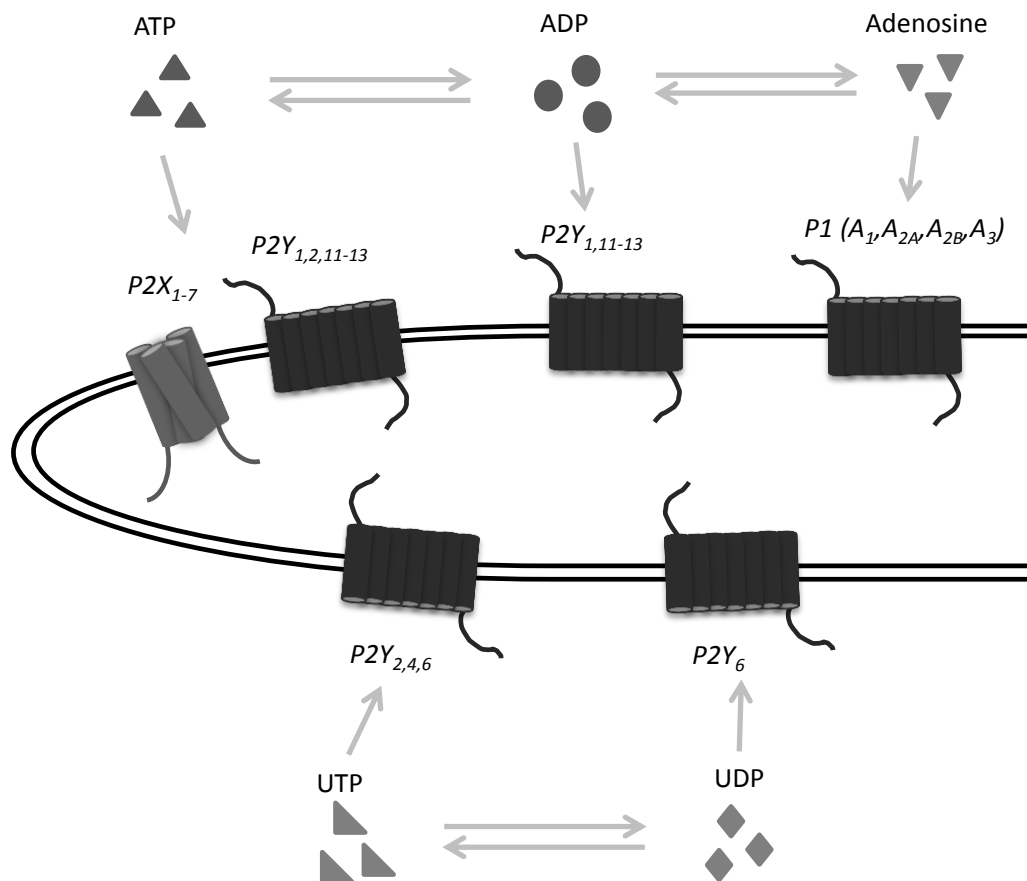


Figure 32. Schematic illustration of purinoceptors interacting with ATP and UTP and their enzymatic breakdown products. P2X receptors are ionotropic channels. P2Y and P1 receptors are metabotropic G-protein coupled receptors (GPCRs). ATP broadly interacts with P2X and P2Y receptors subtypes. Of the P2Y receptors, ATP has specific affinity for P2Y_{1,2,11-13}. ADP is specific for P2Y_{1,11-13} and adenosine activates all P1 receptors subtypes (A₁, A_{2A}, A_{2B} and A₃). UTP is specific for P2Y_{2,4,6} receptors and its break-down product, UDP, is selective for P2Y₆ receptors. ATP and UTP may undergo progressive hydrolysis through ADP and UDP, AMP and UMP, to adenosine and uridine.

4.1.1 PURINORECEPTORS AND VISCERAL PAIN

Following iontophoretic application to human skin and in the human blister base model, ATP elicits pain (Bleehen & Keele, 1977; Hamilton, Warburton, Bhattacharjee, Ward, & McMahon, 2000). The breakdown products of ATP, including adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP) and adenosine, also all evoke algogenic responses in man (Bleehen & Keele, 1977; Coutts, Jorizzo, Eady, Greaves, & Burnstock, 1981). As well as nociceptive signalling, ATP is involved in mechanosensation, inflammation and in the control of vascular tone (Burnstock, 1999; Coutts et al., 1981; Hamilton et al., 2000). In visceral organs, ATP is released from the mucosal epithelium during distension and activates sub-epithelial visceral afferent nerves through specific purinoceptors, most notably P2X₃ (Burnstock, 1999). This purinergic signalling mechanism leads to the transduction of mechanosensation to the central nervous system and the activation of pain pathways. Indeed, distension of human and guinea-pig ureter, rat lung and colon, and mouse urinary bladder, have all led to the pressure-dependent release of ATP (Calvert et al., 2008; Knight, Bodin, De Groat, & Burnstock, 2002; Rich, Douillet, Mahler, Husain, & Boucher, 2003; Vlaskovska et al., 2001; Wynn et al., 2003). In the ureter, release of ATP began at the uroteric pressure threshold for pain (25-30 cm H₂O; (Risholm, 1954)). Specifically, both sub-epithelial fibroblasts isolated from rat intestinal villi and human intestinal epithelial cells are capable of releasing ATP (Dezaki, Tsumura, Maeno, & Okada, 2000; Furuya, Sokabe, & Furuya, 2005). In addition to mechanosensory transduction, the release of ATP from immune

cells and during cell damage implicates purinergic signalling in nociceptive pathways during inflammation, injury and/or infection (Burnstock, 2009a).

The identification of the ligand-gated non-selective P2X₃ ion channel in 1995 led to the proposal that these receptors 'transduce ATP-evoked nociceptor activation' (C. C. Chen et al., 1995; Lewis et al., 1995). Since then numerous studies have shown the importance of homomeric P2X₃, and heteromeric P2X_{2/3} receptors in pain processing and transduction (Wirkner et al., 2007). Indeed, P2X₃ receptor expression correlates well with a nociceptive phenotype in sensory neurones (IB₄ positive and capsaicin-sensitive) (C. C. Chen et al., 1995; Lewis et al., 1995) and is also extensively present in human sub-epithelial sensory nerves (Calvert et al., 2008).

P2X₃ receptors are expressed by 36% of mouse colonic sensory neurones (Brierley, Carter, et al., 2005). This provides parity with functional responses by the selective P2X₃ agonist, α,β -methylene-ATP (α,β -meATP), which excites visceral pelvic and splanchnic nerves; an effect that can be blocked by P2X antagonists including PPADS and TNP-ATP (Brierley, Carter, et al., 2005; Rong & Burnstock, 2004). These responses are potentiated by ARL-67156, an ATPase inhibitor, limiting the break-down of ATP (Wynn et al., 2003) and by the synergistic actions of other mediators including 5-HT, capsaicin and protons (Wynn & Burnstock, 2006). Inhibition of P2X₃ receptors by TNP-ATP can also attenuate acute visceral pain behaviours following intraperitoneal injection of acetic acid (Honore et al., 2002). As well as an involvement in direct activation, pelvic afferent responses to distension are attenuated in the presence of P2X receptor antagonists (Wynn et al., 2004; Wynn et al., 2003), suggesting that P2X

receptors contribute significantly to the transduction of noxious mechanical stimuli in the gut. This is confirmed by *in vivo* models, where P2X₃ -/- mice possess significantly reduced visceromotor reflexes (VMR) during colorectal distension (CRD), in conjunction with the loss of zymosan-induced colonic hypersensitivity (Shinoda et al., 2009). Indeed, single-fibre recordings of afferent responses to circumferential stretch are unaffected by the loss of P2X₃, but mechanical hypersensitivity induced by inflammatory soup was attenuated (Shinoda et al., 2009); further highlighting the role of P2X₃ receptors in the sensitisation of visceral afferents. Pharmacological inhibition of P2X₃ with TNP-ATP also reversed chronic visceral hypersensitivity in a rat acetic acid model of IBS (Xu, Shenoy, Winston, Mittal, & Pasricha, 2008). These findings suggest that P2X₃ receptors contribute significantly in visceral afferent activation to noxious colorectal distension and mechanical hypersensitivity induced by inflammation.

Whilst there exists compelling evidence for an ATP-mediated mechanotransduction pathway, P2X receptors are not mechanically-gated cation channels as described in previous chapters. The release of ATP, its diffusion and subsequent binding to ligand-gated purinoceptors, is a relatively slow mechanism of mechanosensitivity. As such, it is unlikely that P2X receptors act to directly gate mechanical stimuli, but rather ATP is probably better considered a key mediator responsible for the mechanosensory transduction of shear stresses associated with the viscous drag of passing fluids (Burnstock, 1999), and with the acute and chronic sensitisation of afferents during inflammation and injury. This is confirmed by increased release of ATP during colitis following distension in rodent models (Rong et al., 2002; Shinoda

et al., 2009). P2X₃ receptor expression is also up-regulated following chronic visceral inflammation in rat (Xu et al., 2008), and in the enteric nerves of inflamed intestines from inflammatory bowel disease (IBD) patients, suggesting greater influence of this receptor on afferent sensitivity (Yiangou et al., 2001).

As mentioned above, P2Y receptors also significantly contribute to the activation and modulation of sensory neurones by ATP. In addition to the established direct effects of P2Y receptor activation, afferent activation by ATP in P2X_{2/3} and P2X₃ knock-out mice indicates additional signalling pathways for ATP-mediated effects (Rong et al., 2009; Shinoda et al., 2009). The role of other purinoceptors in visceral nociception is poorly understood, with a number of potential receptor candidates capable of mediating these effects expressed on sensory neurones. These include the metabotropic P2Y receptors and P1 receptors (A₁, A_{2A}, A_{2B} and A₃) for the ATP break-down product, adenosine. The expression of these receptors and their involvement in nociceptive signalling is explained in more detail below (Table 7).

Purinergic receptor subtype	Expression in sensory neurones		
	Small	Medium	Large
P2X ₃	***	-	-
P2Y ₁	***	*	-
P2Y ₂	**	*	*
P2Y ₄	*	*	***
P2Y ₆	<i>No evidence</i>		
P2Y ₁₁	<i>No rodent ortholog</i>		
P2Y ₁₂	**	-	-
P2Y ₁₃	**	*	*
P2Y ₁₄	**	*	*
A ₁		***	
A _{2A}		*	
A _{2B}		*	
A ₃	-	-	-

Table 7. Summary of expression of relevant purinergic receptors in sensory neurones. ***high expression, **medium expression and *low expression. For adenosine receptors A₁, A_{2A} and A_{2B}, expression relative to cell size is not known.

4.1.2 P2Y RECEPTORS

P2Y receptors are G-protein coupled receptors that modulate neuronal activity by interactions with other receptors and ion channels. It is likely that P2Y receptors contribute significantly to the regulation of ATP-mediated afferent excitation in the gut; as such the eight P2Y receptor subtypes characterised in humans with their varying nucleotide and ligand sensitivities, as well as their couplings to intracellular signalling cascades, are detailed below (Table 8).

Type	Ligands	Intracellular Coupling
P2Y ₁	ATP, ADP, ADP-β-S, 2-MeSADP, MRS2365	↑PLC/G _{q,11}
P2Y ₂	ATP, UTP, PSB1114	↑PLC/G _{q,11}
P2Y ₄	UTP	↑PLC/G _{q,11}
P2Y ₆	UTP, UDP	↑PLC/G _{q,11}
P2Y ₁₁	ATP, ADP, 2-MeSATP	↑PLC/G _{q,11} & ↑AC/G _s
P2Y ₁₂	ATP, ADP, ADP-β-S, 2-MeSADP	↓AC/G _i
P2Y ₁₃	ATP, ADP, ADP-β-S, 2-MeSADP	↓AC/G _i
P2Y ₁₄	UDP-glucose	↓AC/G _i

Table 8. The selectivity of P2Y receptor ligands and coupling to intracellular signalling pathways. ↑PLC/G_{q,11}, positively coupled to phospholipase C via G_{q,11} proteins; ↓AC/G_i, negatively coupled to adenylyl cyclase via G_i proteins; ↑PLC/G_{q,11} & ↑AC/G_s, positively coupled to both phospholipase C and adenylyl cyclase via G_{q,11} and G_s proteins; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; ADP-β-S, adenosine 5'-O-(2-thiodiphosphate); 2-MeSADP, 2-methylthio ADP; MRS2365, [(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-Amino-2-(methylthio)-9*H*-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl)methyl] diphosphate; UTP, uridine 5'-triphosphate; PSB1114, 4-Thiouridine-5'-O-(β,γ-difluoromethylene)triphosphate; UDP, uridine 5'-diphosphate; 2-MeSATP, 2-methylthio ATP. Summarised from (Gerevich & Illes, 2004).

In contrast to the relatively restricted expression of P2X₃ receptors in small DRG neurones, P2Y receptors are found much more ubiquitously across the size range of sensory neurones. Considerable evidence for the expression of those P2Y receptors in sensory neurones is outlined below. In addition to expression on sensory neurones, certain P2Y receptor subtypes are expressed by other cell types present in neuronal tissues, including satellite glial cells, astrocytes, oligodendrocytes and spinal cord microglia (Fam, Gallagher, & Salter, 2000; Salter & Hicks, 1994).

4.1.3 EXPRESSION OF P2Y RECEPTORS IN SENSORY NEURONES

4.1.3.1 P2Y₁

RNA transcripts for P2Y₁ have been shown to be expressed in rat and mouse dorsal root ganglia (DRG) neurones, rat trigeminal ganglia (TG) and nodose ganglia (NG) (Moriyama et al., 2003; Ruan & Burnstock, 2003; Sanada et al., 2002; Tominaga et al., 2001). In situ hybridisation (ISH) techniques show approximately 15-20% of neurones express P2Y₁ in mouse DRG (Kobayashi et al., 2006; Moriyama et al., 2003; Xiao et al., 2002). This expression is neuronal-specific and is not seen in Schwann or satellite cells and is also not seen in large cells (cell area >1200µm²), with an equal distribution across unmyelinated and thinly-myelinated neurones (Kobayashi et al., 2006; Nakamura & Strittmatter, 1996). Immunohistochemical (IHC) staining of DRG, TG and NG in rat neurones labelled approximately 80% of small neuronal populations (Fong, Krstew, Barden, & Lawrence, 2002; Ruan & Burnstock, 2003). In agreement with ISH studies, 34-45% of these showed colocalisation with NF200 (i.e. neurofilament positive) neurones (Ruan & Burnstock, 2003). Importantly, 80% of small

neurones coexpress, not only both the nociceptive markers P2X₃ and TRPV1, but also P2Y₁ receptors (Gerevich et al., 2004). This correlates well with a 73-84% colocalisation of P2Y₁ with P2X₃ receptors (Ruan & Burnstock, 2003). Finally, approximately 25% of P2Y₁ immunoreactive sensory neurones co-stain for CGRP and 62% of P2Y₁ immunoreactive neurones were IB4 positive, which concurs with the reported high colocalisation with P2X₃ (Ruan & Burnstock, 2003).

4.1.3.2 P2Y₂

P2Y₂ RNA transcripts are expressed in mouse DRG, rat DRG and rat TG and NG (Moriyama et al., 2003; Ruan & Burnstock, 2003; Sanada et al., 2002). ISH expression of P2Y₂ mRNA transcripts have been reported in as high as 77% of rat DRG neurones, across both small and large cell sizes (Molliver et al., 2002). However, multiple studies in mouse have shown considerably lower expression (~25%) (Kobayashi et al., 2006; Moriyama et al., 2003). This may be a genuine species difference or possibly a discrepancy caused by differences in the methodology between the studies. IHC staining of cat DRG shows 35% immunoreactivity (IR) for P2Y₂, specifically in small-diameter neurones (Ruan et al., 2005). P2Y₂ IR in large diameter cell bodies is punctate and does not colocalise with P2X₃, however a significant proportion of small-diameter neurones do colabel for P2Y₂ and P2X₃ receptors (Mo, Peleshok, Cao, Ribeiro-da-Silva, & Séguéla, 2013). Of P2Y₂ mRNA expressing neurones, approximately a third coexpressed TRPV1 mRNA (Moriyama et al., 2003), also suggesting a nociceptive phenotype.

4.1.3.3 P2Y₄

The expression of P2Y₄ in rat DRG, NG and TG has been confirmed by RT-PCR (Ruan & Burnstock, 2003; Sanada et al., 2002). However, using ISH techniques, Kobayashi *et al.* failed to find expression of P2Y₄ mRNA in DRG cell bodies (Kobayashi et al., 2006). In contrast, single-cell RT-PCR suggests modest expression in sensory neurones (see below) (X. Chen, Molliver, & Gebhart, 2010). The P2Y₄ receptor has been observed by immunohistochemical labelling, mainly in larger neurones (~70%), with some expression in small sensory neurones (Ruan & Burnstock, 2003). In terms of characterising the P2Y₄-IR population in DRG, 97% of neurones co-express NF200, 10% co-express CGRP and 2% co-express IB₄.

4.1.3.4 P2Y₆

RT-PCR suggests the presence of P2Y₆ in rat sensory ganglia (Ruan & Burnstock, 2003; Sanada et al., 2002), although ISH to P2Y₆ mRNA did not label any sensory neurones (Kobayashi et al., 2006). At the present time, no IHC expression data exists confirming the expression of P2Y₆ in sensory neurones.

4.1.3.5 P2Y₁₁

The P2Y₁₁ receptor was originally cloned from human placenta and P2Y₁₁ mRNA has been identified in the human spleen and HL-60 cells (Communi, Govaerts, Parmentier, & Boeynaems, 1997). However, no rodent ortholog has yet been reported for P2Y₁₁.

4.1.3.6 P2Y₁₂, P2Y₁₃ and P2Y₁₄

P2Y₁₂, P2Y₁₃ and P2Y₁₄ were identified in DRGs by RT-PCR (Malin & Molliver, 2010). ISH of P2Y₁₂, P2Y₁₃ and P2Y₁₄ mRNA revealed localisation in glial cells closely associated with neurones (Kobayashi et al., 2006). However, IHC showed intense staining of all three receptors in small DRG neurones, with P2Y₁₃ and P2Y₁₄ observed in some larger neurones (Malin & Molliver, 2010). As yet, the expression profiles of these receptors remains unclear.

Clearly contradictions in the quantification of expression levels exist in the literature, predominantly between ISH and IHC techniques. Given the close homology of the P2Y family, the specificity of an antibody for a particular subtype must be very critically assessed. This is especially true of polyclonal antibodies that bind likely multiple homologous P2Y epitopes, which the vast majority of the studies that are outlined above rely on. Whilst these discrepancies may be due to the tools used to probe sensory ganglia, the possibility of extensive post-transcriptional/pre-translational regulation could also account for differences between the reported expression levels of mRNA versus protein. Collectively, the data does suggest considerable expression of P2Y receptors on sensory neurones, especially P2Y₁ and P2Y₂, which are both positively coupled to phospholipase C through G_{q,11} protein and are likely to potentiate excitatory afferent responses. This does not discount other P2Y receptor subtypes in sensory signalling, but does suggest their actions may be indirect and/or involve more complex multicellular pathways.

4.1.3.7 Expression in visceral extrinsic sensory nerve populations

To date, only two studies have examined expression of P2Y receptors in visceral sensory nerves. Using single-cell RT-PCR of lumbosacral bladder-projecting DRG neurones, Chen *et al.* showed that 47% and 22% express P2Y₂ and P2Y₄ receptors, respectively. In thoracolumbar DRG levels, these were reduced to 38% and 13% for P2Y₂ and P2Y₄ receptors, respectively (X. Chen et al., 2010). There is also evidence of P2Y₁ and P2Y₂ receptor expression in kidney-projecting sensory neurones (H. Wang, Wang, & Galligan, 2010).

4.1.4 P2Y RECEPTORS IN MODULATION OF SENSORY NERVE FUNCTION

The expression of P2Y receptors on sensory nerves correlates with a complex range of functional effects. P2Y receptors are capable of evoking action potential firing and inducing intracellular calcium flux. Indeed, P2Y₁ and P2Y₂ receptor activation depolarises isolated sensory neurones and increases their excitability to current injection (Yousuf et al., 2011). The P2Y₁, P2Y₁₂ and P2Y₁₃ agonist ADP, can excite TG neurones and can also induce intracellular Ca²⁺ release from small IB4 positive DRG neurones (Borvendeg et al., 2003; Ceruti et al., 2008). In contrast to other sensitising effects, whole-cell patch-clamping recordings indicate that P2Y₁ receptors can actually inhibit P2X₃ receptors in DRG neurones (Gerevich, Müller, & Illes, 2005), which provide a further desensitising feedback pathway regulating ATP-mediated signalling.

Behaviourally, P2Y₁-mediated increase in intracellular calcium is required for inflammatory hyperalgesia in intraplantar CFA models (Andó, Méhész, Gyires, Illes, & Sperlág, 2010; Malin & Molliver, 2010). In addition, the P2Y₁ receptor

subtype has been shown to transduce ATP-dependent mechanical stimuli and to have modulatory effects on TRPV1 activity (Nakamura & Strittmatter, 1996; Tominaga et al., 2001). Finally, normal thermal sensation requires P2Y₁ receptors (Molliver, Rau, McIlwrath, Jankowski, & Koerber, 2011; Yajima, Sato, Giron, Nakamura, & Mizumura, 2005). This effect is, at least partially, mediated by TRPV1 phosphorylation following P2Y₁ activation (Kwon et al., 2014). The contribution of P2Y₁ receptors is increased following inflammation, where up-regulation of P2Y₁ receptors is responsible for normal heat sensitisation in cutaneous nociceptors (Jankowski et al., 2012).

P2Y₂ receptor agonists can also evoke excitatory effects in sensory neurones. Indeed, the P2Y₂ and P2Y₄ ligand UTP, can induce calcium influx in small nociceptive neurones (Molliver et al., 2002). In cutaneous fibre recordings, UTP can excite nociceptive fibres, whilst the selective P2Y₆ ligand, UDP, failed to do so (Molliver et al., 2002; Stucky et al., 2004). UTP is also capable of depolarising neurones and triggering action potentials in current-clamp experiments (Ceruti et al., 2008; Molliver et al., 2002; Sanada et al., 2002). Whilst this effect was observed irrespective of neuronal size, suggesting that P2Y₂ and/or P2Y₄ receptors may be present across a range of small and large neurones (Sanada et al., 2002), a mechanical hypersensitivity in cutaneous single fibre recordings following UTP application was only observed in a subset of C-fibre nociceptors (Lechner & Lewin, 2009). This does however fit with expression patterns observed by ISH and IHC (Mo et al., 2013; Molliver et al., 2002). Functional implications of the UTP activation of large neurones are yet to be determined.

P2Y₁₂ antagonism can attenuate both inflammatory pain behaviours and hypersensitivity evoked by partial sciatic nerve ligation in mice (Andó et al., 2010; Kobayashi et al., 2008). In a different model of neuropathic pain, following P2Y₁₂ receptor antagonism and in P2Y₁₂ -/- mice, tactile allodynia was significantly reduced (Tozaki-Saitoh et al., 2008). However, in isolated neurones, activation of G_i-coupled P2Y₁₂, P2Y₁₃ and P2Y₁₄ receptors actually inhibits excitatory signalling (Malin & Molliver, 2010). These effects, in contradiction to the inhibitory actions reported by Malin *et al.* in neurones, are mediated by non-neuronal cells in the spinal cord, most probably microglia (Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008). In addition to P2Y₁₂ receptors, microglia are also modulated by P2Y₆, P2Y₁₃ and P2Y₁₄ receptors (Kobayashi, Yamanaka, Yanamoto, Okubo, & Noguchi, 2012). This supports the effects of intrathecal injections of UTP and UDP in neuropathic pain models which, in contrast to reported stimulatory effects of UTP *in vitro*, were both capable of reducing mechanical sensitivity and inhibiting induced tactile allodynia (Okada, Nakagawa, Minami, & Satoh, 2002).

The behavioural changes and modulation of neuronal sensitivity by P2Y receptor ligands are the result of intracellular coupling to neuronal ion channels, including voltage-gated calcium, sodium and potassium channels. Specifically, inwardly rectifying K⁺ channels (K_{ir}3), M-type K⁺ channel (K_V7), voltage-activated calcium channels (Cav2) and Ca²⁺-dependent K⁺ channels (K_{Ca}2), are all examples of ion channels known to be coupled to P2Y receptors (Filippov et al., 2004; Gerevich et al., 2004; Schicker et al., 2010; Zaika, Tolstykh, Jaffe, & Shapiro, 2007). In addition, nucleotide agonists can alter both TTX-

sensitive and TTX-resistant (TTX-R) sodium currents in dorsal root ganglia (DRG) neurones (Park et al., 2004). Hyperpolarising shifts by ADP and UTP in the conductance-voltage relationship of the slowly-inactivating TTX-R sodium current will likely affect nociceptor sensitivity (Baker, 2005; Baker et al., 2003; Park et al., 2004). Potentiation of TRPV1 responses to capsaicin, protons and heat by P2Y₂ receptor activation in rat DRG neurones also contributes to the sensitising effects of ATP (Moriyama et al., 2003; Tominaga et al., 2001). Indeed, thermal hyperalgesia in mice to ATP injection was not lost following deletion of TRPV1 or P2Y₁, however injection of UTP was capable of recapitulating a similar phenotype, suggesting P2Y₂ involvement (Moriyama et al., 2003).

Collectively these findings show that P2Y receptors are extensively expressed by sensory neurones and act to mediate aspects of thermal, acute and neuropathic nociception. However, the data does provide an indication of the complexity of P2Y signalling intraneuronally, with multiple receptors acting synergistically to regulate neuronal function. Moreover, the role of P2Y receptors on non-neuronal cell types and the ability to regulate ATP signalling is apparent.

4.1.5 ROLE OF P2Y RECEPTORS IN VISCERAL AFFERENT SIGNALLING AND THE ENTERIC NERVOUS SYSTEM

Evidence for P2Y receptor signalling in visceral afferents is observed in projections to the bladder and kidney. Specifically, activation of P2Y₂ receptors sensitise P2X₂, P2X₃ and TRPV1 currents present in these neurones, contributing to afferent excitability and hypersensitivity (X. Chen et al., 2010; H. Wang et al., 2010). In the context of the bladder, increased afferent firing is associated with urinary frequency, urgency and pain (Nazif, Teichman, &

Gebhart, 2007). In the kidney, local release of ATP could modulate TRPV1 function and act to regulate the release of vasodilator peptides, increasing glomerular filtration rate and sodium/water excretion (H. Wang et al., 2010). In addition, this also likely contributes to pain associated with occlusions of the kidney ducts.

Further to the extrinsic visceral afferent function, P2Y receptors are also found in enteric neurones, where they contribute to secretomotor reflexes within the submucosal plexus. For example, P2Y receptors (and P1 receptors, which will be discussed later) are involved in the modulation of mechanosensitive 5-HT release, fluid, mucin and ion (Cl^- , HCO_3^- & K^+) secretion, amongst other functions (Christofi, 2008; Christofi et al., 2004; Hu et al., 2003). Whether these activities impact on visceral afferent sensitivity or visceral nociceptive pathways remains unclear. As mentioned in the previous chapter, little evidence exists for crosstalk between extrinsic afferent projections and the enteric nervous system, at least with respect to mechanosensitivity.

4.1.6 P1 RECEPTORS

Adenosine is an important neuromodulator in the central nervous system (CNS) and the periphery, acting through P1 receptor subtypes: A_1 , A_{2A} , A_{2B} and A_3 receptors. In addition to its release by sensory and sympathetic neurones, endothelial cells, immune cells, fibroblasts and mast cells, the dephosphorylation of ATP by ectonucleotidases also yields adenosine (Sawynok & Liu, 2003). Within the CNS, adenosine facilitates both excitatory and inhibitory actions on neuronal function and the central inflammatory processes.

Specifically in pain transmission, adenosine can act at spinal, supraspinal and peripheral sites to induce both pronociceptive and antinociceptive effects (Sawynok, 1998). As mentioned previously, adenosine applied at peripheral targets induces pain in somatic (Bleehen & Keele, 1977; Pappagallo et al., 1993) and visceral models (Crea et al., 1990; Gaspardone et al., 1995). By contrast, activation of adenosine A₁ receptors in the spinal cord by intrathecal injection of adenosine produces analgesia (Belfrage, Segerdahl, Arnér, & Sollevi, 1999; Eisenach, Curry, & Hood, 2002). In peripheral nociceptive afferent fibres, adenosine can inhibit or augment firing rates dependent on the actions of adenosine A₁ or A_{2A} receptors (Aley, Green, & Levine, 1995; Taiwo & Levine, 1990). Differential coupling of adenosine receptors to neuronal effectors, such as ion channels and intracellular signalling pathways, enables adenosine to modulate these contrasting effects on neuronal excitability.

4.1.7 EXPRESSION OF P₁ RECEPTORS IN SENSORY NEURONES

In line with the functional effects of adenosine, adenosine receptors have a wide expression distribution across multiple tissues, including neuronal and non-neuronal cell types in the CNS, spinal cord and cardiovascular tissues (Marala & Mustafa, 1998), as well as in skeletal muscle (Lynge & Hellsten, 2000) and at motor nerve terminals (Baxter, Vega-Riveroll, Deuchars, & Parson, 2005). Relevant to afferent nociceptive pathways, adenosine receptors are expressed by the somata of sensory neurones. Selective adenosine receptor subtype expression within different tissues mediates the contrasting algogenic and analgesic effects of adenosine.

In sensory neurones, these effects are transduced predominantly by adenosine A₁ receptors and to some extent A_{2A} and A_{2B} receptors. Indeed, RT-PCR of whole DRG reveals significant expression of adenosine A₁ receptor compared to minimal levels of adenosine A_{2A} and A_{2B} receptor mRNA transcripts. No expression of adenosine A₃ receptors was detected in DRG (L. Li et al., 2010). Quantitatively, in viscerally-projecting DRG neurones, single-cell RT-PCR revealed approximately 80% of TRPV1-positive cells also expressed adenosine A₁ receptors. In these same cells, adenosine A_{2A} and A_{2B} receptor mRNA transcripts were detected in less than 22% of cells, whilst no cells expressed adenosine A₃ receptors (Ru, Surdenikova, Brozmanova, & Kollarik, 2011). Immunostaining has also identified adenosine A₁ receptors in cultured TG neurones and weak adenosine A_{2A} receptor expression in mice DRG neurones (Carruthers et al., 2001; L. Li et al., 2010). In contrast to adenosine A₁ receptors, mRNA for adenosine A_{2A} receptor appears not to be present in neurones of the spinal cord (Kaelin-Lang, Lauterburg, & Burgunder, 1998).

4.1.8 P1 RECEPTORS IN MODULATING SENSORY AFFERENT FUNCTION

Adenosine released in the periphery exerts effects on nociceptive processing by the activation of the four adenosine receptor subtypes (A₁, A_{2A}, A_{2B} and A₃), which in turn are coupled to multiple intracellular signalling pathways. Of relevance to afferent function are the opposing effects of adenosine A₁ and A_{2A} receptor activation, resulting in decreased or increased intracellular cAMP and opposing changes in neuronal excitability (Aley et al., 1995; Khasar et al., 1995; Taiwo & Levine, 1990). Whilst electrophysiological studies show that A₁ receptor agonists can increase sensory afferent firing innervating the lung

(Hong, Ho, Kwong, & Lee, 1998), knee joint (Dowd, McQueen, Chessell, & Humphrey, 1998) and mesenteric afferent neurones (Kirkup, Eastwood, Grundy, Chessell, & Humphrey, 1998), adenosine-mediated peripheral mechanical hyperalgesia is significantly regulated by A_{2A} receptor activation (L. Li et al., 2010). Interestingly, adenosine can also directly inhibit TRPV1 Ca²⁺ flux, independent of A_{2A} receptor activation, and may serve as a means of regulating excessive TRPV1 activation and nociceptor excitability during hyperalgesic states (Puntambekar, Van Buren, Raisinghani, Premkumar, & Ramkumar, 2004).

In the enteric nervous system, adenosine receptors are expressed differentially along the GI tract and in neuronal and non-neuronal tissues. In both the submucosal plexus and the myenteric plexus, all four receptors are detectable by RT-PCR, with lower expression of adenosine A₁ and A_{2A} receptors compared to adenosine A_{2B} and A₃ receptors (Christofi et al., 2001). This is confirmed by immunostaining against adenosine A₃ receptors in substance P-positive submucosal ganglia. By contrast, A_{2B} receptors are expressed predominantly in vasoactive intestinal peptide (VIP)-ergic neurones, with A_{2A} receptors split between the two populations (Christofi et al., 2001). In addition, adenosine may evoke release of secondary mediators from non-neuronal cells within the GI tract, including mast cells (Olah & Stiles, 1995). The modulation of afferent function by these paracellular influences is unclear and poorly studied.

Given that multiple purinoceptors, including P2Y and P1 receptors, are expressed by sensory neurones innervating the viscera and somatic tissues, it is likely that they contribute significantly to the ability of ATP to sensitise

afferents during inflammation and injury. As shown in previous chapters, voltage-gated sodium channel subtype 1.9 (Nav1.9) contributes greatly to afferent chemosensitivity, mechanosensitivity and mechanical hypersensitivity during acute inflammatory stimulation. It is likely, therefore, that purinoceptors may work in conjunction with Nav1.9 to regulate afferent sensitivity and excitability.

4.1.9 MODULATION OF Nav1.9 BY PURINOCEPTORS

The current mediated by Nav1.9 can be up-regulated in sensory somata by activation of G-protein coupled intracellular signalling pathways (Baker, 2005; X. Chen et al., 2011; Maingret et al., 2008; Rush & Waxman, 2004). Specifically, activation of GPCRs by inflammatory mediators, including extracellular ATP, can increase peak Nav1.9 current amplitude in a concentration-dependent manner (Baker, 2005). Such up-regulation of Nav1.9 current can lead to moderate neuronal depolarisation and increased afferent excitability. In contrast, Maingret *et al.* were unable to evoke Nav1.9 current enhancement when ATP was applied independently, but could drive current up-regulation with GTP- γ -S and also with an inflammatory cocktail of mediators (bradykinin, PGE₂, histamine, norepinephrine & ATP) (Maingret et al., 2008). Therefore they propose Nav1.9 represents a coincidence detector to inflammatory mediators in nociceptors. The exact mechanism by which ATP, either on its own or in the presence of other mediators, can cause Nav1.9 current enhancement remains unknown. The possibility that Nav1.9 current potentiation by either ATP-mediated P2Y receptor activation or adenosine-mediated P1 receptor activation could significantly contribute to the effects of both ATP and adenosine on

neuronal excitability is still to be investigated. This may represent an important mechanism by which ATP can act to acutely and chronically sensitise visceral afferents.

4.2 AIMS

It is clear from the studies mentioned above that the ability of ATP to modulate afferent sensitivity and pain pathways is dependent on the actions of multiple purinoreceptors, as well as the regulation of multiple downstream effector channels.

Therefore the aims of this chapter were;

1. To investigate how deletion of Nav1.9 affects colonic afferent excitability to both ATP and its breakdown product, adenosine
2. To study P2Y receptor activation of colonic afferent fibres
3. To investigate how Nav1.9 $-/-$ colonic afferent activity is affected by P2Y receptor activation
4. To understand whether P2Y receptor agonists can excite human mesenteric afferent fibres

4.3 METHODS

4.3.1 WHOLE-NERVE ELECTROPHYSIOLOGICAL RECORDINGS FROM LUMBAR SPLANCHNIC NERVE IN MOUSE DISTAL COLON

Multi-unit activities were recorded from the whole lumbar splanchnic nerve in a tubular preparation of the distal colon, as described in detail in Chapter 3. Briefly, the distal colon from either Nav1.9 $+/+$ or Nav1.9 $-/-$ mice was dissected free with associated mesentery and neurovasculature. Field potential recordings from the lumbar splanchnic nerve were made using a borosilicate glass suction electrode, amplified, band-pass filtered (gain 5K; 100-1300Hz) and digitally filtered for 50Hz noise. Raw traces were digitised at 20 kHz and action potential firing counts determined using a threshold set at twice the background noise (typically 100 μ V). Neurogram and pressure traces were displayed on a PC using Spike 2 software (Cambridge Electronic Design, UK). The distal colon was tied to two cannulas using thin thread, and both perfused intraluminally with Kreb's buffer at 0.1ml/min and superfused with Kreb's buffer at 7-8ml/min. The pressure was set to baseline and then a \sim 2mmHg end pressure applied before allowing the preparation to stabilise for approximately 45 minutes.

4.3.2 PROTOCOLS FOR TUBULAR PREPARATION OF MOUSE COLON

After the stabilising period, drugs were applied by bath superfusion of a 20ml volume. Purinergic agonists (ATP [0.1, 1 & 3 mM], ADP [0.1, 1 & 3mM], UTP [0.1, 1 & 3mM], UDP [1, 3 & 10mM] and adenosine [0.1, 0.3, 1 & 3 mM]) were applied in separate experiments, while different concentrations of the same agonist were applied consecutively in most cases. Where repeat concentrations of drugs

were given, a 60 minute interval was maintained or until activity had stabilised to baseline. For antagonist studies, using P2X receptor antagonists, PPADS (30 μ M) and TNP-ATP (10-30 μ M) and adenosine P1 receptor antagonist, CGS 15943 (3 μ M), repeat applications of ATP (1mM or 3mM) were applied. Once stable responses to ATP application were achieved, the antagonist (PPADS, TNP-ATP or CGS 15943) was applied both immediately prior to the next ATP application (by bath superfusion of 20mL volume) and during the ATP application. Later, antagonist studies using selective P2Y receptor antagonists, MRS2500 (3 μ M), Ticlopidine (100 μ M) and AR-C118925X (10 μ M) were applied to separate naive preparations prior to, and in the presence of, a singular agonist concentration (ATP [1mM], ADP [1mM] or UTP [1mM]). Selective P2Y receptor agonist studies using MRS2365 (10 μ M) and PSB1114 (3-10 μ M) were undertaken in separate preparations.

4.3.3 WHOLE-NERVE ELECTROPHYSIOLOGICAL RECORDINGS FROM MESENTERIC NERVE OF HUMAN RESECTED BOWEL TISSUES

As described in the previous chapter, resected human colon was obtained after full written consent from patients undergoing elective surgery at Barts Health NHS Trust, London after approval by the local Research Ethics Committee (NREC 10/H0703/71). Macroscopically normal colon, appendix and ileum were obtained from patients undergoing colectomy as part of their normal surgical treatment for bowel cancer. Specimens were removed away from the tumour, resection margins or lymphatic drainage field and were surplus to histopathological assessment. Details of the tissues used are described in Table 9. Once removed, tissues were immediately placed in cold carbogenated Kreb's

buffer prior to sharp dissection of full-thickness colonic wall (~4cm square with mesenteric fan down the centre) and depending on length, the appendix was split in two or used in its entirety. Tissue was superfused with oxygenated Krebs's buffer (supplemented with 10 μ M nifedipine and 10 μ M atropine) at a rate of 7mL/min and at a temperature of 32-34°C. Under a dissection microscope, the neurovascular bundles in the associated mesentery were blunt dissected and mesenteric nerves identified and cleared of connective tissue. Electrophysiological recordings were subsequently made in an identical manner to that of animal tissues.

#	Tissue	Disease	Operation	Age	Sex
1	Appendix	Cancer	Right hemicolectomy	86	M
2	Sigmoid colon	Cancer	Laparoscopic anterior resection	59	F
3	Sigmoid colon	Cancer	Anterior resection	52	M
4	Sigmoid colon	Cancer	Laparoscopic anterior resection	50	F
5	Appendix	Cancer	Right hemicolectomy	62	F
6	Appendix	Cancer	Laparoscopic right hemicolectomy	81	F
7	Ileum	Cancer	Laparoscopic right hemicolectomy	76	M
8	Appendix	Cancer	Subtotal colectomy	57	M
9	Sigmoid colon	Cancer	Anterior resection	76	M
<i>Mean age / M:F ratio</i>				<i>67</i>	<i>1.25:1</i>

Table 9. Patient details of human tissue used in mesenteric nerve recordings

4.3.4 PROTOCOLS FOR HUMAN BOWEL TISSUE RECORDINGS

In human tissue experiments, concentrations of ATP (1 and 10mM), ADP (1 and 10mM) and UTP (1 and 10mM) were applied by bath superfusion of a 20ml volume sequentially at 1 hour intervals, in variable total bath volumes dependent on tissue type. As such, comparisons between relative nucleotide activities should not be over-interpreted.

4.3.5 CHROMOGENIC *IN SITU* HYBRIDISATION

Retrograde labelling of colonic tissues was achieved following the same protocol as outlined in Chapter 2. In brief, Fast Blue (2% in saline) was injected into the wall of the distal colon of naïve C57BL6 mice following laparotomy under general anaesthetic. Mice were allowed to recover and dorsal root ganglia (T13 and L1) were harvested after three days following transcardial 4% paraformaldehyde perfuse fixation. DRGs were post-fixed (2hr, 4% PFA) and cryoprotected (30% sucrose) before freezing at -80°C. Cryostat sections (10µm) were collected sequentially across 40 slides per DRG, with *N*=5 animals per slide.

Sections were prepared as required for use in RNAscope 2-plex (Chromogenic) Detection Kit (Advanced Cell Diagnostics, Inc., USA). The chromogenic *in situ* hybridisation assay employs two independent signal amplification systems to detect RNA-specific probes hybridised to the tissue. Presence of the target RNA transcripts was detected by punctate dots of two distinctly coloured chromogen precipitates (red and green). Sections were washed in 0.1M PBS followed by 10 minute endogenous enzyme block (against horseradish peroxidase (HRP) and

alkaline phosphatase (AP)) at room temperature (RT). Antigen retrieval was performed by incubation at ~100°C in 'Pretreat 2' (a sodium citrate buffer) for 15 minutes and 40 minutes at 40°C in 'Pretreat 3', a protease digestion step. These steps were optimised empirically in order to provide the maximal chromogenic signal whilst minimising structural tissue degradation. The two target probes consist of 6-10 short oligonucleotide pairs, covering approximately 50 bases of the target mRNA per oligonucleotide, these were hybridised for 2 hours at 40°C. The *SCN11A* probe targets NM_011887.3, 349-1283bp within exon 3 –exon 10 and the *P2Y₁* probe targets NM_008772.4, 1101-2092bp. Probe design and selection was carried out by ACD (Mm-SCN11A-C1 (catalog #403531) and Mm-P2ry1-C2 (catalog #406061-C2). Once hybridised, six amplification steps were performed, culminating in the binding of HRP- and AP-labelled probes. Addition of chromogenic substrates produced green and red coloured products, respectively. Sections were counterstained (50% haemotoxylin and 0.02% ammonia water), dried (60°C, 15mins) and coverslipped in EcoMount (BioCare Medical, USA) mounting medium.

4.3.6 DATA ANALYSIS

In electrophysiological multi-unit recordings, peak changes and time profiles of nerve activity were determined by subtracting baseline firing (5 minutes before drug application) from increases in nerve activity following drug superfusion. Changes in nerve activity were compared between Na_v1.9+/+ and Na_v1.9-/- animals using Student's t-test or two-way ANOVA with Bonferroni post-hoc test, as appropriate.

From the time profiles, six criteria were established, these are outlined in detail in Figure 33 and the associated figure legend. *Time to onset, time to peak, time to 80% response, time to 50% response* and *time to return to baseline* of time profiles were compared between genotypes by 2-way ANOVA with Bonferroni's post-hoc, as appropriate. In antagonist studies featuring repeat application of ATP, paired two-tailed t-tests were used to compare responses to ATP prior to, after and in the presence of antagonists (PPADS, TNP-ATP and CGS 15943). In later antagonist studies, unpaired t-tests compared existing agonist responses (ATP, ADP and UTP) with independent responses, in the presence of an antagonist (MRS2500, Ticlopidine and AR-C118925X). All data are expressed as mean \pm S.E.M. $P < 0.05$ was taken to indicate statistical significance.

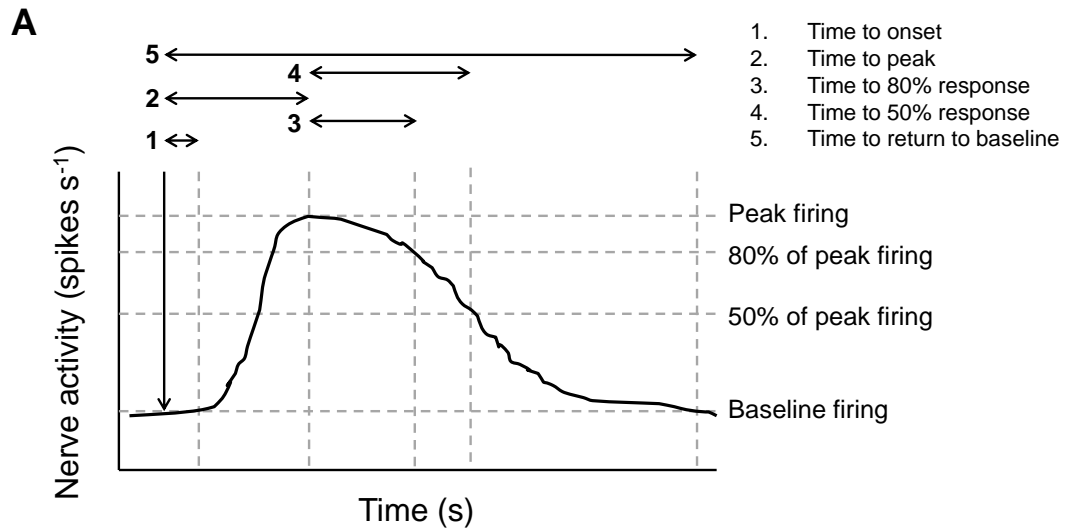


Figure 33. Representative time profile of response to drug application and data derived from time profiles. The *baseline firing* was established as the mean nerve activity of at least 3 minutes prior to drug application. *Peak firing* was determined as the peak nerve firing minus the baseline activity. (1) *Time to onset* was determined as the time taken for firing to increase above baseline after superfusion of drug into the bath began. (2) *Time to peak* represents the time taken from drug entering the bath to activity reaching its peak. (3) *Time to 80% response* and (4) *time to 50% response* were determined as the time taken for nerve activity, after peaking, to reduce to 80% and 50% of the maximal, respectively. (5) *Time to return to baseline*, represents the time taken for nerve activity to recover fully to baseline after drug application.

4.3.7 IMAGING AND QUANTITATION

The percent of area covered by reaction products in *in situ* hybridisation experiments for *Nav1.9* mRNA and *P2Y₁* mRNA were determined for all Fast Blue positive (FB+) DRG cells (ImageJ 1.45S analysis software, NIH, USA). Fast Blue and brightfield images at 20x magnification were taken of every 10th sequential DRG section (sampling at ~100µm intervals). The two chromogenic reaction products (green and red) were sufficiently divergent in chromaticity to enable reliable and robust isolation from RGB colour images (Adobe Photoshop CS3, USA). Specifically, all other colour hues (orange, yellow, blue and violet) were desaturated and the background was subtracted, with only red and green pixels corresponding to the presence of *Nav1.9* mRNA and *P2Y₁* mRNA, respectively, remaining. Fast Blue-labelled cells were identified using ImageJ software and these overlays transposed onto brightfield images. Percent area covered of reaction products per FB+ cell was subsequently quantified. A background measure was taken from a comparable area size of the image containing no tissue, and subtracted from percent area covered of FB+ cells. Quantified reaction product of non-soma axon tissue regions was used as a threshold to determine positive labelling (~3%) and as the limitations of the detection method. This equated to 1-3 chromogen dots per approximately equivalent area of 10 cells; with anything with greater expression than this was deemed to be positively labelled.

4.3.8 DRUGS

Adenosine 5'-triphosphate (ATP) disodium salt hydrate, adenosine 5'-diphosphate monopotassium salt hydrate (ADP), uridine 5'-triphosphate

trisodium salt dehydrate (UTP) uridine 5'-diphosphate disodium salt hydrate (UDP) were all purchased from Sigma-Aldrich (UK) and made up to a stock of 300mM in water. Also purchased from Sigma-Aldrich were nifedipine (100mM: DMSO), atropine (100mM: EtOH) and indomethacin (30mM: DMSO). 2'-3'-O-(2,4,6-Trinitrophenyl)adenosine-5'-triphosphate tetra(triethylammonium) salt (TNP-ATP), pyridoxalphosphate-6-azophenyl-2'-4'-disulfonic acid tetrasodium salt (PPADS), [[(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-Amino-2-(methylthio)-9*H*-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt (MRS2365), (1*R**,2*S**)-4-[2-Iodo-6-(methylamino)-9*H*-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (MRS2500), 5-[(2-Chlorophenyl)methyl]-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridine hydrochloride (Ticlopidine) and 4-Thiouridine-5'-O-(β,γ -difluoromethylene)triphosphate tetrasodium salt (PSB1114) were diluted in water and CGS 15943 was diluted in DMSO; all were purchased from Tocris Biosciences (UK). AR-C118925X was diluted in DMSO and was a kind gift from Novartis UK. All compounds were diluted in Krebs' buffer to appropriate working concentrations on the day of use.

Where possible, concentrations were chosen based on their use in comparable *ex vivo* afferent nerve preparations in the literature (such as jejunal mesenteric nerve, bladder pelvic nerve or oesophageal vagal nerve; see Table 10.). If this strategy was not possible, then IC₅₀ and EC₅₀ concentrations at the receptor of interest were used to estimate an appropriate concentration in the colonic afferent preparation. Based on the existing literature, this was typically a 10-100x increase in concentration from isolated cell protocols (Wynn & Burnstock,

2006; Wynn et al., 2004). Wash-out of drug effect, repeat application and concentration-dependent effects by increasing 10-fold increments, were all used to confirm specific pharmacology in pilot studies.

Drug	Concentration used in current study (mM)	Previous experiments	Concentration used in previous study (mM)	Reference
ATP	0.1, 1, 3	Colonic pelvic nerve	0.1 - 10	(Wynn & Burnstock, 2006; Wynn et al., 2003)
ADP	0.1, 1, 3			
UTP	0.1, 1, 3			
UDP	1, 3, 10			
Adenosine	0.1, 0.3, 1, 3	Oesophageal vagal nerve	0.1	(Ru et al., 2011)
PPADS	0.03	Splanchnic and pelvic nerves	0.03 - 0.1	(Brierley, Carter, et al., 2005; Rong et al., 2009; Wynn et al., 2003)
TNP-ATP	0.01 – 0.03	Bladder pelvic nerve	0.03	(Rong et al., 2002)
CGS 15943	0.003	IC ₅₀ – adenosine-stimulated adenylate cyclase assay in synaptoneurosome	0.000003 – 0.000020 (depending on receptor subtype)	(M. Williams et al., 1987)
MRS2365	0.01	EC ₅₀ - inositol phosphates assay in COS-7 cells	0.0000004	(Chhatriwala et al., 2004)
MRS2500	0.003	IC ₅₀ – ADP-stimulated platelet aggregation	0.00000095	(Cattaneo et al., 2004)
Ticlopidine	0.1			
PSB1114	0.003-0.01	EC ₅₀ – inositol phosphates assay in astrocytoma 1321N1 cells	0.000134	(El-Tayeb, Qi, Nicholas, & Müller, 2011)
AR-C118925X	0.01	IC ₅₀ – ATP-γS-induced mucin secretion	0.001	(Kemp, Sugar, & Jackson, 2004)

Table 10. Rationale for concentrations of purinergic agonists and antagonists used in the present studies. Where agonists or antagonists have been used in equivalent *ex vivo* afferent nerve preparations, these concentrations were applied to the present studies. If no comparable studies exist in the literature, then the EC₅₀ or IC₅₀ in a cellular assay was used to estimate an appropriate concentration for application in afferent preparations; usually 10-100x greater than the EC₅₀ or IC₅₀.

4.4 RESULTS

4.4.1 COLONIC SPLANCHNIC AFFERENT NERVE RESPONSES TO ATP IN *NAV1.9* *+/+* AND *NAV1.9* *-/-* MICE

To investigate how Nav1.9 affects purinergic signalling pathways in colonic visceral afferents, whole-nerve recordings of responses to ATP superfusions were made in a cannulated tubular preparation of the distal colon. Sequential application of increasing ATP concentrations (0.1mM, 1mM & 3mM) in Nav1.9 *+/+* mice led to concentration-dependent increases in afferent activation (see Figure 34). Following 1mM and 3mM ATP application, a significant direct excitation was evoked, however, following 0.1mM ATP application, a transient (~3mins) decrease in baseline activity was observed, followed by a modest increase in afferent activity (Figure 34C). Changing the order of these sequential ATP applications still evoked comparable responses. In Nav1.9 *-/-* mice, responses to ATP were attenuated at all concentrations examined ($p < 0.0001$; Figure 34F). Baseline firing prior to ATP application did not differ significantly between genotypes ($p = 0.19$; Figure 35A). The time taken from ATP entering the bath to peak firing (*Time to peak*) and the time taken for an increase in firing to occur above baseline (*Time to onset*) were not significantly longer in Nav1.9 *-/-* preparations ($p = 0.38$ and $p = 0.74$, respectively; Figure 35B). Perhaps unsurprisingly given the reduced peak firing in Nav1.9 *-/-* mice, the times taken for firing to return back to 80% and 50% of maximum firing and the time taken to return to baseline following ATP application (*Time to 80% response*, *Time to 50% response* and *Time to return to baseline*) were significantly reduced at all concentrations apart from 0.1mM ($p < 0.01$, $p < 0.01$ and $p < 0.01$, respectively).

Even at the highest concentration of ATP tested (3mM), firing rates and nerve terminal sensitivity were reduced by 73% in Nav1.9 -/- afferents. This suggests that ATP is unable to evoke the same degree of afferent excitation in the absence of Nav1.9.

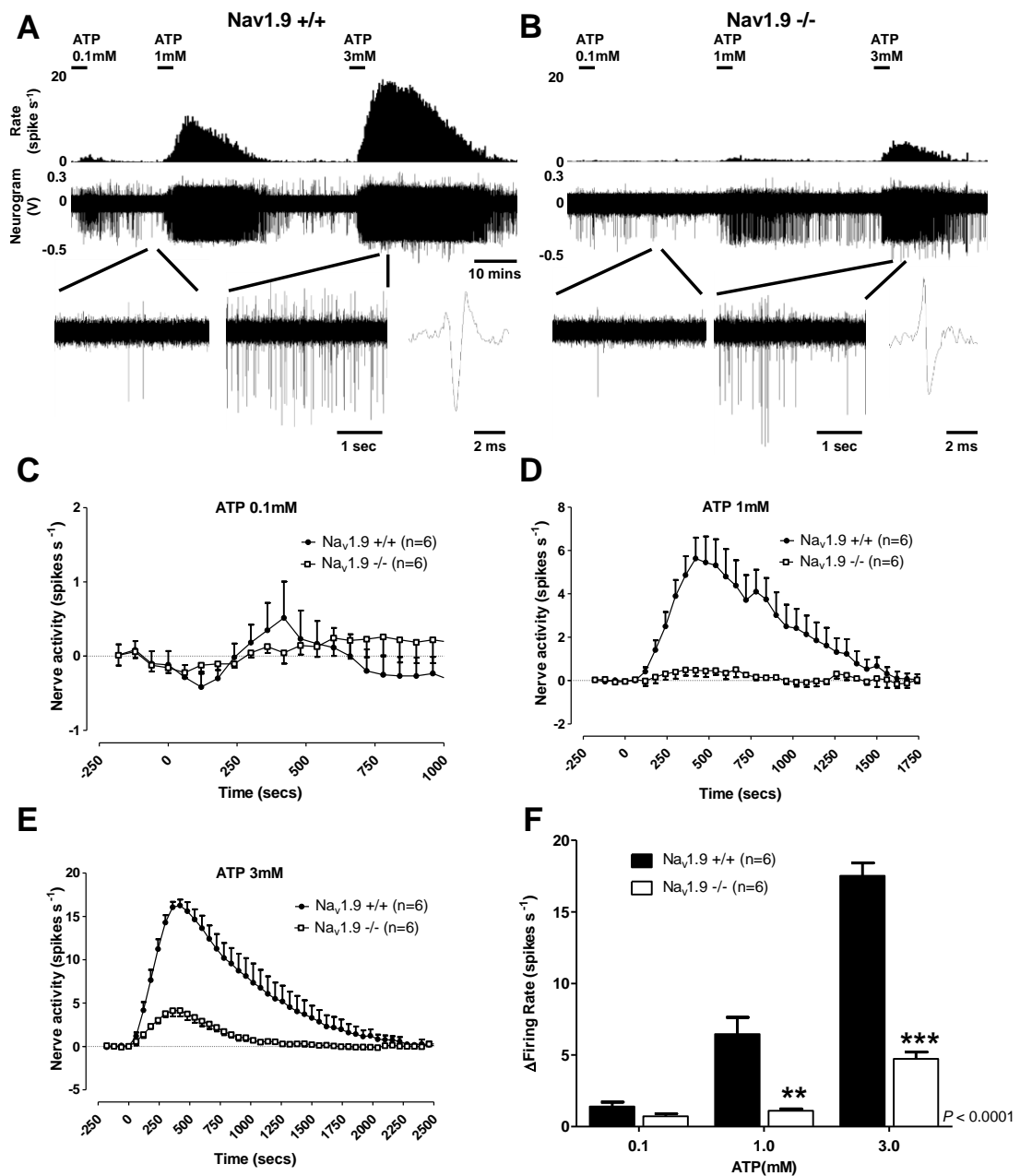


Figure 34. Effect of increasing concentrations of ATP on colonic splanchnic nerve activity in Nav1.9 +/+ and Nav1.9 -/- mice. Example raw histogram and associated neurogram of nerve activity in Nav1.9 +/+ (A) and Nav1.9 -/- (B) mice to 0.1mM, 1mM and 3mM ATP. Below, expanded traces of activity at baseline and following addition of 3mM ATP and example action potential for each genotype. Response profiles to addition of 0.1mM ATP (C), 1mM ATP (D) and 3mM ATP (E) in both Nav1.9 +/+ and Nav1.9 -/- mice. F Peak increase in nerve activity following addition of 0.1mM, 1mM & 3mM ATP above baseline activity in Nav1.9 +/+ and Nav1.9 -/- mice ($p < 0.0001$, 2-way ANOVA with Bonferroni's post-hoc, ** $p < 0.01$, *** $p < 0.001$).

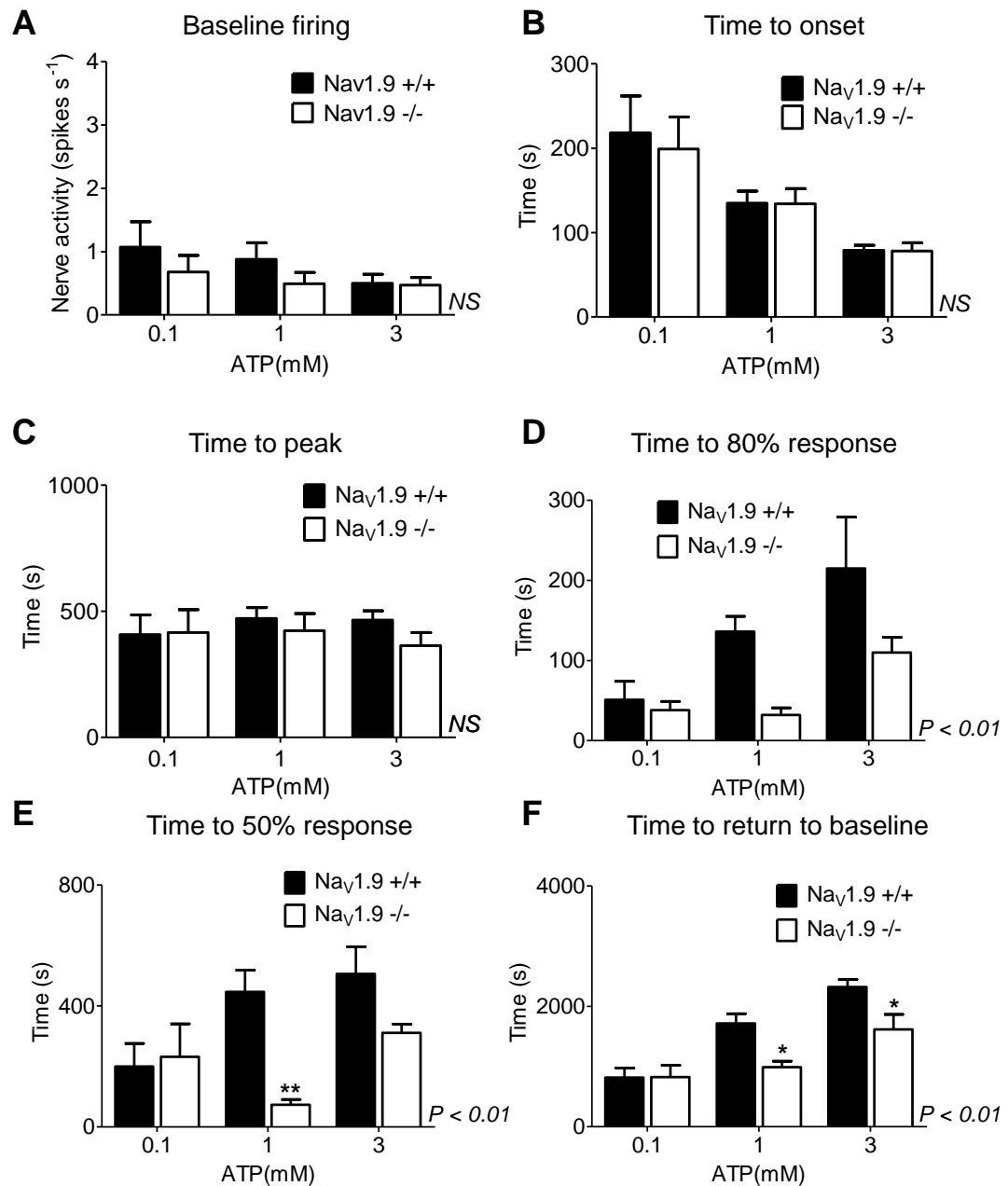


Figure 35. Baseline firing prior to ATP application and duration of responses to ATP in colonic splanchic nerve. A Mean nerve activity over 3 minutes prior to application of ATP in Nav1.9 +/+ and Nav1.9 -/- ($N = 6$, both groups; $p = 0.19$, 2-way ANOVA). B Time taken from ATP superfusion entering the recording bath to an increase in mean nerve activity above baseline ($N = 6$, both groups; $p = 0.74$, 2-way ANOVA). C Time taken from ATP entering the recording bath to reach peak nerve activity ($N = 6$, both groups; $p = 0.38$, 2-way ANOVA). D Time taken for nerve activity to return to 80% of peak response ($N = 6$, both groups; $p < 0.01$, 2-way ANOVA). E Time taken for nerve activity to return to 50% of peak response ($N = 6$, both groups; $p < 0.01$, 2-way ANOVA). F Time taken for nerve activity to return to baseline ($N = 6$, both groups; $p < 0.01$, 2-way ANOVA).

4.4.2 P2X RECEPTOR AND P1 ANTAGONISM OF ATP-MEDIATED ACTIVATION OF COLONIC SPLANCHNIC AFFERENT NERVE IN Nav1.9 +/+ MICE

To further investigate the purinergic signalling pathways responsible for ATP-mediated excitation of colonic afferents, cannulated distal colon preparations from wild-type Nav1.9 +/+ mice were pre-incubated with antagonists for P2X receptors and adenosine P1 receptors. Repeat applications of ATP evoked stable responses at both 1mM and 3mM concentrations. Addition of the P2X antagonist, PPADS (30 μ M) or the selective P2X_{1,2/3,3} antagonist, TNP-ATP (10-30 μ M), to repeat concentrations of 1mM ATP in Nav1.9 +/+ animals produced no significant change in activity (PPADS: $p = 0.23$; TNP-ATP: $p = 0.49$; Figure 36). Even at an increased concentration of 30 μ M TNP-ATP, no change in afferent activity to 1mM ATP was observed ($N = 2$). Following repeat applications of 3mM ATP, pre-incubation with the P2X receptor antagonists, PPADS (30 μ M) or TNP-ATP (10 μ M) resulted in a significant reduction in afferent activity ($p < 0.05$ and $p < 0.05$, respectively; Figure 36). Pre-incubation with the non-selective P1 (adenosine) receptor antagonist CGS 15943 (3 μ M) to repeat applications of 3mM ATP led to a 28.5% reduction in afferent firing ($p < 0.05$). Given that complete inhibition of responses to ATP were not achieved by supramaximal concentrations of either P2X receptor or non-specific P1 adenosine receptor antagonists, we investigated the role of P2Y receptors in colonic afferent activation.

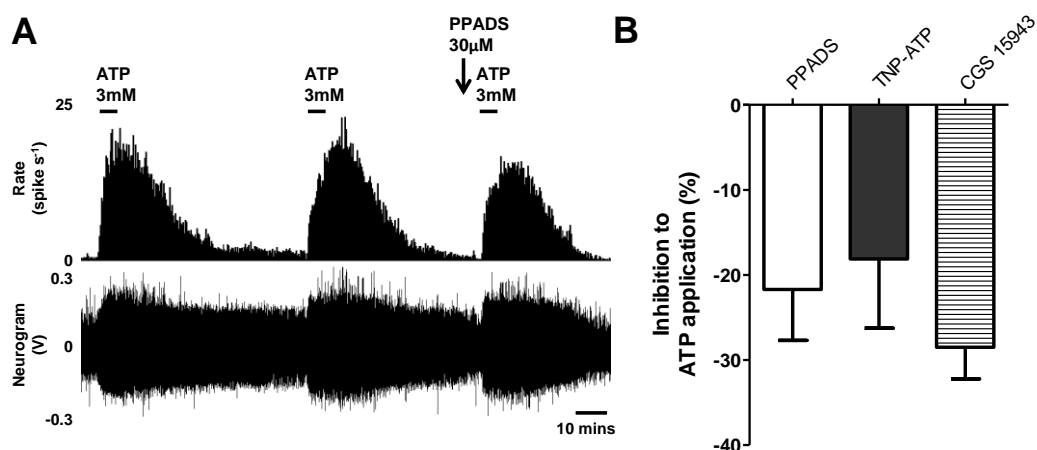


Figure 36. Effect of P2X receptor and P1 (adenosine) receptor antagonism by PPADS, TNP-ATP and CGS 15943 on colonic splanchnic nerve activity to ATP. A Example rate histogram and raw neurogram of nerve activity following repeat application of 3mM ATP prior to and following addition of 30μM PPADS. B Percent reduction in peak activity to application of 3mM ATP following pre-incubation with 30μM PPADS ($N = 3$), 10-30μM TNP-ATP ($N = 3-5$) and 3μM CGS 15943 ($N = 3$).

4.4.3 COLONIC SPLANCHNIC AFFERENT NERVE RESPONSES TO P2Y LIGANDS IN *Nav1.9 +/+* AND *Nav1.9 -/-* MICE

Using the P2Y receptor agonists, ADP (selective for P2Y₁, P2Y₁₂ and P2Y₁₃ receptors) and UTP (selective for P2Y₂, P2Y₄ and P2Y₆ receptors) the role of P2Y receptor-mediated excitation in *Nav1.9 +/+* and *Nav1.9 -/-* animals was evaluated. In *Nav1.9 +/+* tissues, both ADP and UTP elicited concentration-dependent increases in afferent firing, although in both instances responses at 0.1mM were modest (Figure 37 & Figure 39). The response to ADP was moderately larger than that of ATP in terms of peak, although the duration of response was equivalent (e.g. *Time to return to baseline*: ATP 3mM, 2322 ± 125 s; ADP 3mM, 2217 ± 384 s; $p = 0.80$, two-tailed unpaired t-test; Figure 41A). The response to UTP at equivalent concentrations was significantly smaller than both ADP and ATP, and was considerably shorter in duration (e.g. *Time to return to baseline*: UTP 3mM, 1337 ± 113 s; vs ATP, $p < 0.001$, two-tailed unpaired t-test; Figure 41A). Responses to ADP were significantly reduced in *Nav1.9 -/-* mice, ($p < 0.001$; Figure 37), as were responses to UTP ($p < 0.001$; Figure 39). Interestingly, in contrast to baseline firing prior to application of both ATP and UTP, a significant difference was observed in baseline firing rates of ADP in the absence of *Nav1.9* (Figure 38 & Figure 40). As observed for ATP, *Time to peak* and *Time to onset* of ADP application were not different between genotypes (Figure 38B & C). However, following ADP application *Time to 80% response*, *Time to 50% response* and *Time to return to baseline* were all significantly attenuated in *Nav1.9 -/-* mice, likely due to not reaching the same degree of peak firing observed in *Nav1.9 +/+* mice.

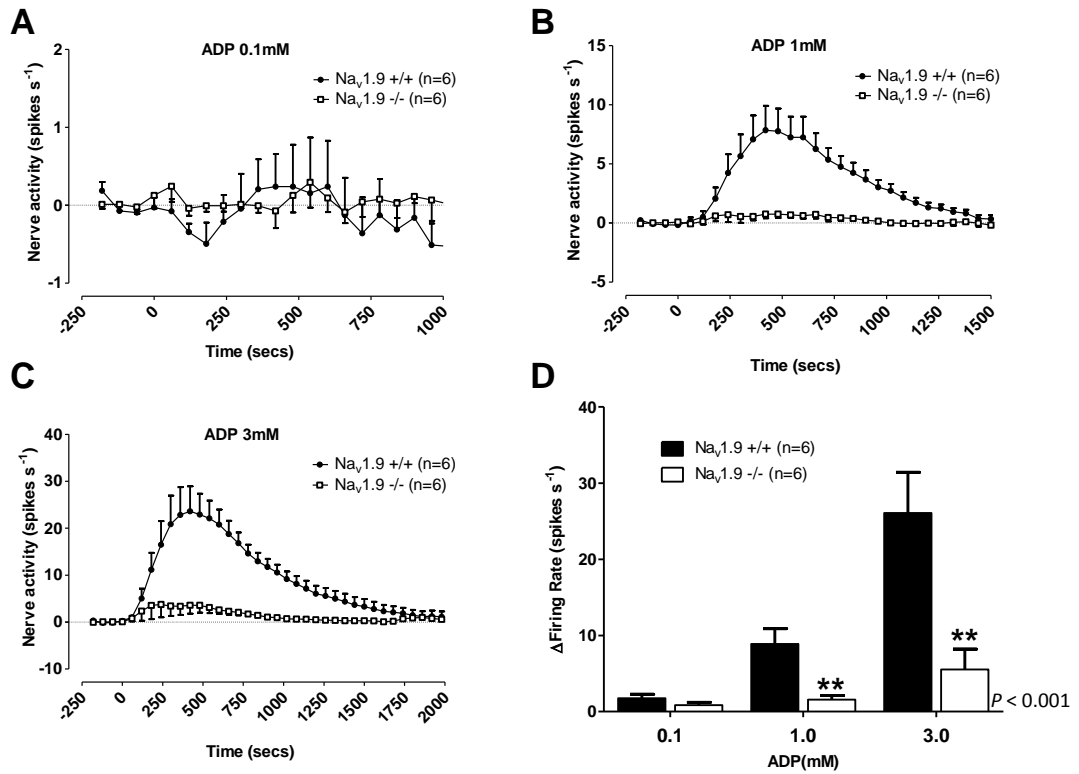


Figure 37. Effect of increasing concentrations of P2Y₁, P2Y₁₂ and P2Y₁₃ agonist, ADP on colonic splanchnic nerve activity in Na_v1.9 +/+ and Na_v1.9 -/- mice. Response profiles for addition of 0.1mM ADP (A), 1mM ADP (B) and 3mM ADP (C) in both Na_v1.9 +/+ and Na_v1.9 -/- mice (*N* = 6, each group). D Peak increase in nerve activity following addition of 0.1mM, 1mM & 3mM ADP above baseline activity in Na_v1.9 +/+ and Na_v1.9 -/- mice (*N* = 6, each group; *p* < 0.001, 2-way ANOVA with Bonferroni's post-hoc, ***p* < 0.01).

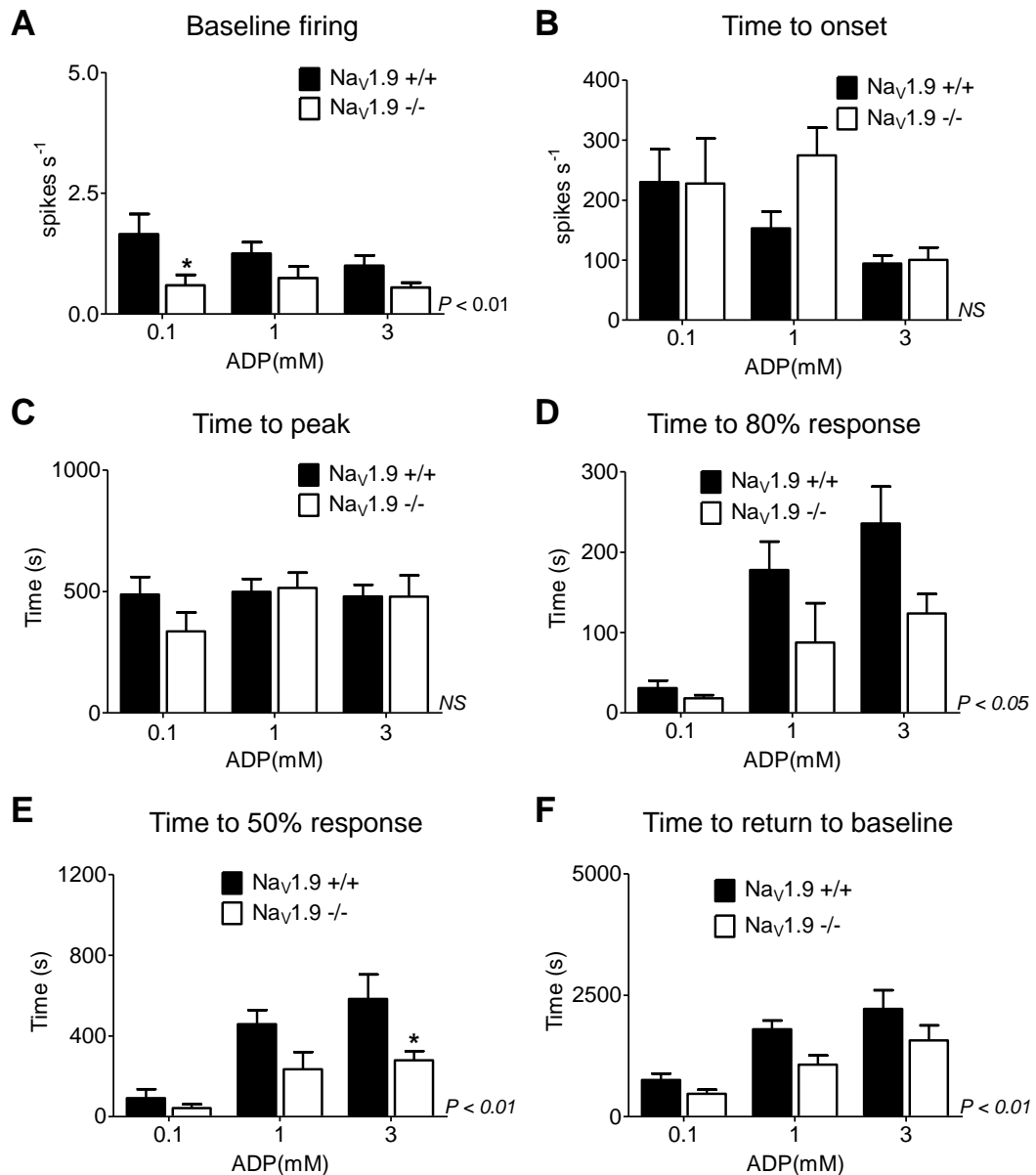


Figure 38. Baseline firing prior to ADP application and duration of responses to ADP in colonic splanchnic nerve. A Mean nerve activity over 3 minutes prior to application of ADP in Na_v1.9 +/+ and Na_v1.9 -/- ($N = 6$, both groups; $p < 0.01$, 2-way ANOVA with Bonferroni's post-hoc, $*p < 0.05$). B Time taken from ADP superfusion entering the recording bath to an increase in mean nerve activity above baseline ($N = 6$, both groups; $p = 0.26$, 2-way ANOVA). C Time taken from ADP entering the recording bath to reach peak nerve activity ($N = 6$, both groups; $p = 0.42$, 2-way ANOVA). D Time taken for nerve activity to return to 80% of peak response ($N = 6$, both groups; $p < 0.05$, 2-way ANOVA). E Time taken for nerve activity to return to 50% of peak response ($N = 6$, both groups; $p < 0.01$, 2-way ANOVA). F Time taken for nerve activity to return to baseline ($N = 6$, both groups; $p < 0.01$, 2-way ANOVA).

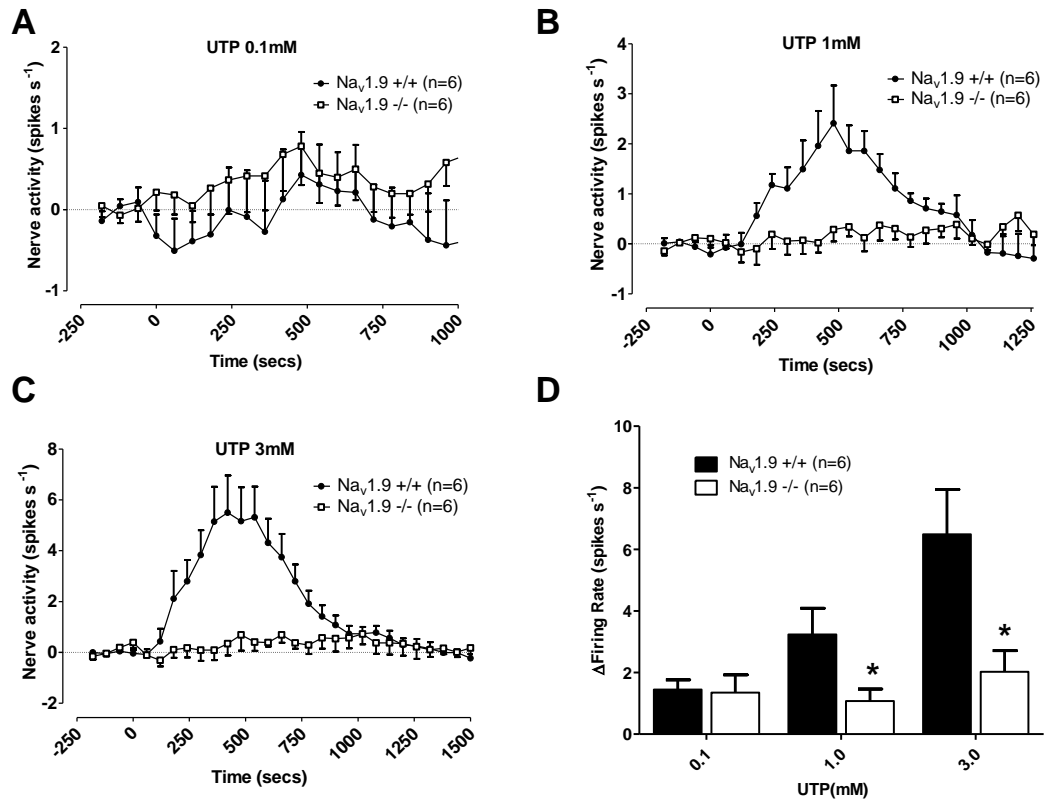


Figure 39. Effect of increasing concentrations of P2Y₂, P2Y₄ and P2Y₆ agonist, UTP on colonic splanchnic nerve activity in Na_v1.9 +/+ and Na_v1.9 -/- mice. Response profiles to addition of 0.1mM UTP (A), 1mM UTP (B) and 3mM UTP (C) in both Na_v1.9 +/+ and Na_v1.9 -/- mice (*N* = 6, each group). D Peak increase in nerve activity following addition of 0.1mM, 1mM & 3mM UTP above baseline activity in Na_v1.9 +/+ and Na_v1.9 -/- mice (*N* = 6, each group; *p* < 0.001, 2-way ANOVA with Bonferroni's post-hoc, **p* < 0.05).

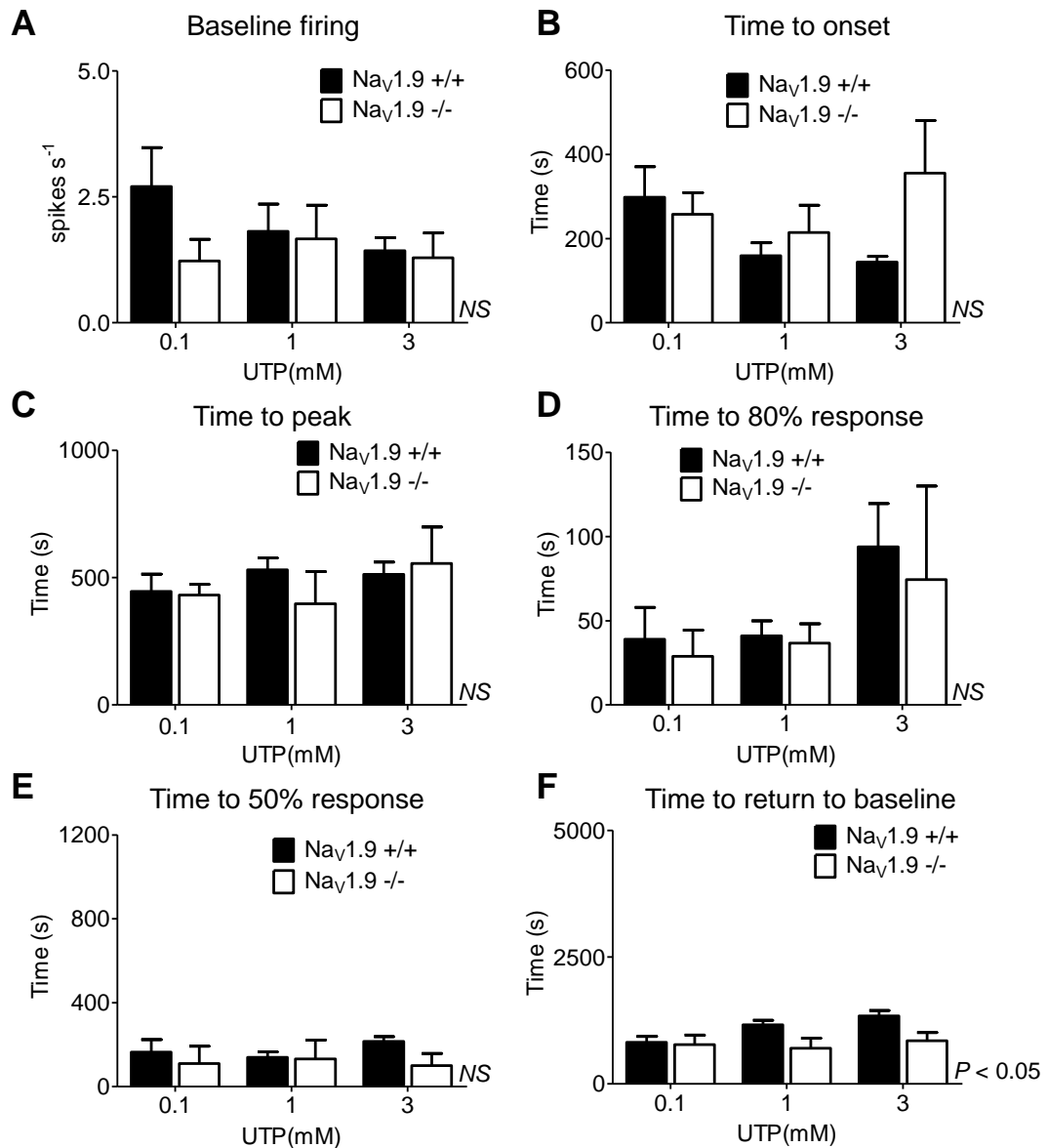


Figure 40. Baseline firing prior to UTP application and duration of responses to UTP in colonic splanchnic nerve. A Mean nerve activity over 3 minutes prior to application of UTP in Na_V1.9 +/+ and Na_V1.9 -/- ($N = 6$, both groups; $p = 0.20$, 2-way ANOVA). B Time taken from UTP superfusion entering the recording bath to an increase in mean nerve activity above baseline ($N = 6$, both groups; $p = 0.19$, 2-way ANOVA). C Time taken from UTP entering the recording bath to reach peak nerve activity ($N = 6$, both groups; $p = 0.64$, 2-way ANOVA). D Time taken for nerve activity to return to 80% of peak response ($N = 6$, both groups; $p = 0.62$, 2-way ANOVA). E Time taken for nerve activity to return to 50% of peak response ($N = 6$, both groups; $p = 0.25$, 2-way ANOVA). F Time taken for nerve activity to return to baseline ($N = 6$, both groups; $p < 0.05$, 2-way ANOVA).

In order to discount a P2Y₆-mediated excitation of colonic afferents by UTP, the selective P2Y₆ receptor agonist, UDP was superfused into the recording bath. At 1mM and 3mM, UDP did not robustly excite colonic afferents in Nav1.9 +/+ mice (Figure 41A). However, at 10mM concentrations UDP did evoke a small increase in afferent firing equivalent to ~17% of a 3mM ATP application (Figure 41B).

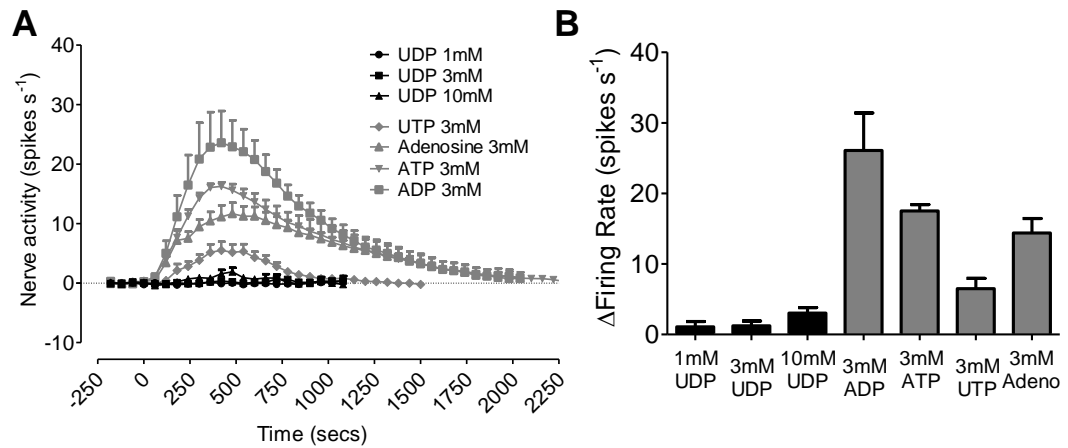


Figure 41. Comparison of colonic afferent activation by UDP with responses evoked by ATP, ADP, adenosine and UTP. A Response profiles for addition of 1mM, 3mM and 10mM UDP in Na_v1.9 +/+ mice ($N = 3$). For comparison and on the same axis, response profiles to 3mM ATP, 3mM ADP, 3mM adenosine and 3mM UTP are also shown ($N = 6$, each agonist). B Peak increase in nerve activity following addition of 1mM, 3mM and 10mM UDP and 3mM ATP, 3mM ADP, 3mM adenosine and 3mM UTP.

4.4.4 EXPRESSION OF P2Y₁ AND Na_v1.9 IN MOUSE COLON-PROJECTING DRG NEURONES BY *IN SITU* HYBRIDISATION

The excitatory responses observed in multi-unit recordings from the splanchnic nerve to ADP and UTP demonstrates significant expression of purinoceptors known to bind these nucleotides (i.e. for ADP, P2Y_{1,11-13} and for UTP, P2Y_{2,4}). To investigate this further, the expression of P2Y₁ and Na_v1.9 in thoracolumbar sensory neurones innervating the colon was determined by chromogenic *in situ* hybridisation (Figure 42). Functional electrophysiological data was recorded from the splanchnic nerve, the sensory afferent cell bodies of which are located in their highest frequency in DRG T13 and L1 (Robinson et al., 2004). Analysis of chromogenic reaction products in retrograde-labelled colonic DRG neurones from these DRG observed P2Y₁-positive expression in $26.9 \pm 5.2\%$ of neurones (Figure 42G). In the same sections, expression of Na_v1.9 was also determined in cells retrogradely labelled with Fast Blue by chromogenic *in situ* hybridisation, of which $56.1 \pm 4.9\%$ expressed Na_v1.9 transcripts. This was comparable with isotopic *in situ* hybridisations for Na_v1.9 which labelled $50.5 \pm 3.3\%$ of FB+ cell bodies (see Chapter 2). In addition, the specificity of chromogenic *in situ* hybridisation for both P2Y₁ and Na_v1.9 was confirmed by negative probe controls (including exclusion of primary probes (Figure 42D & E) and the use of a negative control probe against the *dapB* gene of *Bacillus subtilis*, which is not expressed in mammalian tissues (Figure 42F)). Na_v1.9 and P2Y₁ expression was determined using independent chromogenic reaction products (green and red) showing co-expression in $13.7 \pm 3.8\%$ of colonic sensory neurones (Figure 42G).

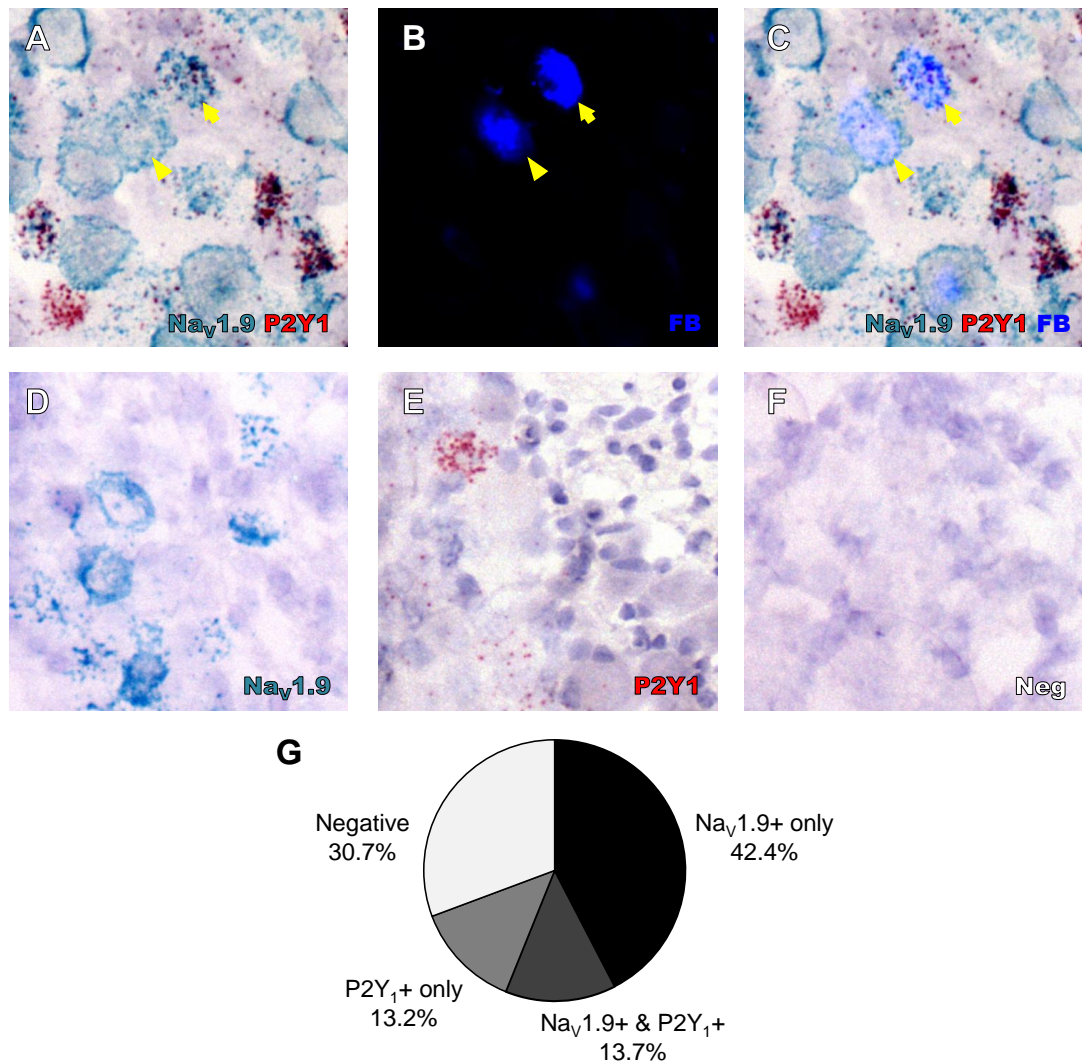


Figure 42. Expression of $P2Y_1$ and $Na_v1.9$ mRNA in mouse colon-projecting DRG neurones by chromogenic *in situ* hybridisation. A Co-expression of $Na_v1.9$ and $P2Y_1$ mRNA transcripts in $Na_v1.9$ $+/+$ mice. B Retrograde Fast Blue (FB) labelling from colon in thoracolumbar DRG section. C Combined $Na_v1.9$, $P2Y_1$ mRNA expression with FB labelling. Yellow arrowhead indicates a colon projecting neurone positive for $Na_v1.9$ but negative for $P2Y_1$ mRNA expression. Yellow arrow indicates a colon projecting neurone positive for both $Na_v1.9$ and $P2Y_1$ mRNA expression. D No primary probe control for $P2Y_1$. E No primary probe control for $Na_v1.9$. F Negative control using bacterial-specific probe against *dabB* from *Bacillus subtilis*. G Relative expression values for $Na_v1.9$ and $P2Y_1$ mRNA in FB-positive colon-projecting neurones ($N = 5$ animals, $n = 181$ FB+ cells).

4.4.5 COLONIC SPLANCHNIC AFFERENT NERVE RESPONSES TO ADENOSINE IN *Nav1.9* *+/+* AND *Nav1.9* *-/-* MICE

Next, the deletion of *Nav1.9* on activation of colonic splanchnic afferents by adenosine was investigated. Adenosine is an enzymatic break-down product the of ectonucleotidase activity on ATP. As such, it is relevant to understand the contribution of adenosine to the overall excitatory response profile caused by ATP in both *Nav1.9* *+/+* and *Nav1.9* *-/-* mice. Adenosine elicited concentration-dependent increases in colonic afferent firing across all concentrations tested in wild-type animals (Figure 43). In *Nav1.9* *-/-* mice, peak responses were significantly attenuated ($p < 0.001$), although the time taken to reach this peak was unaffected ($p = 0.09$; Figure 44C). In agreement with observations for the P2Y ligands, *Time to 80% response*, *Time to 50% response* and *Time to return to baseline* were all significantly reduced in *Nav1.9* *-/-* mice as a result of a reduced peak response at any given concentration ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively; see Figure 44). Baseline firing was significantly different between *Nav1.9* *+/+* and *Nav1.9* *-/-* mice ($p < 0.05$; Figure 44).

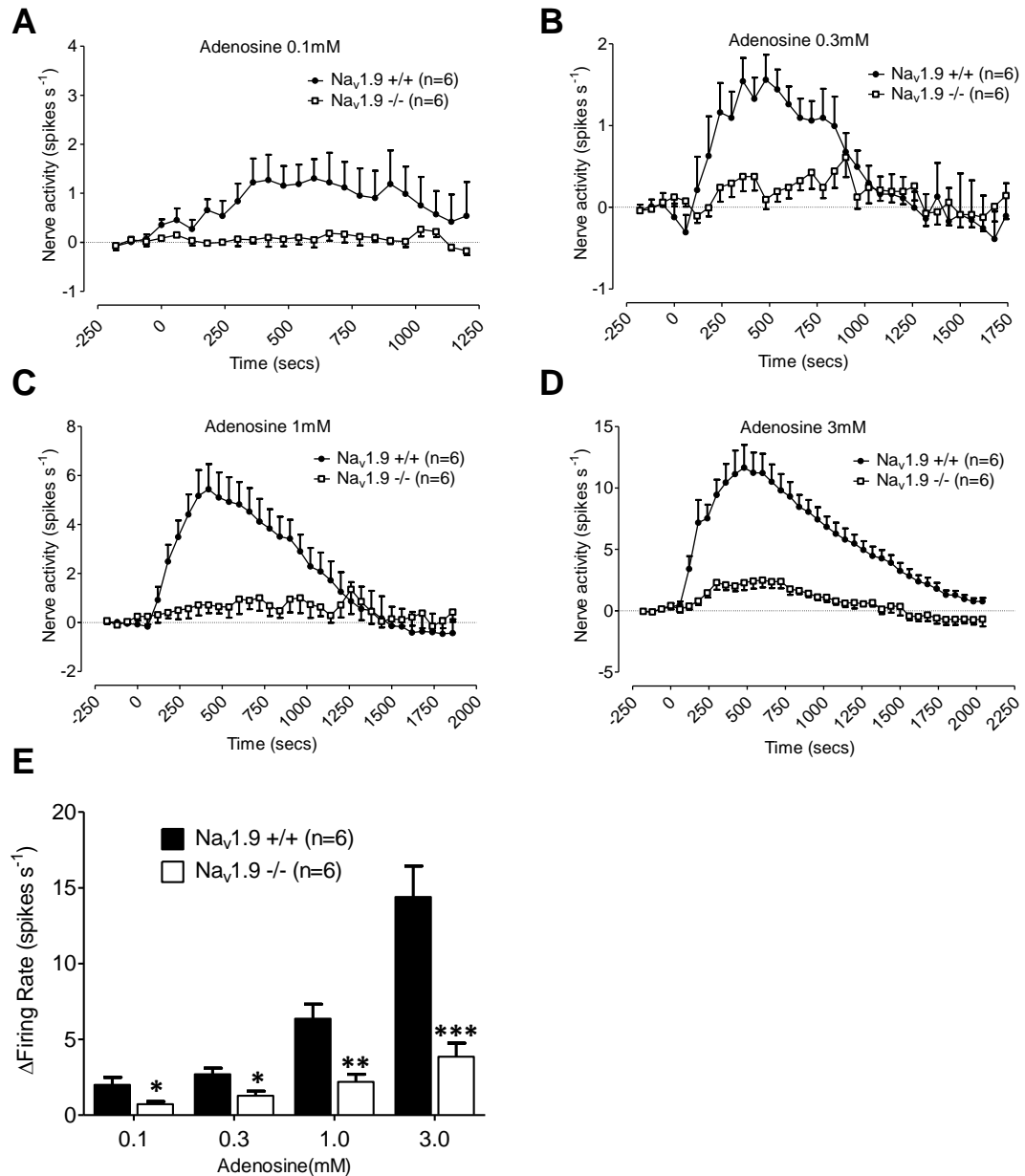


Figure 43. Effect of increasing concentrations of adenosine on colonic splanchnic nerve activity in Na_v1.9 +/+ and Na_v1.9 -/- mice. Response profiles for addition of 0.1mM adenosine (A), 0.3mM adenosine (B), 1mM adenosine (C) and 3mM adenosine (D) in both Na_v1.9 +/+ and Na_v1.9 -/- mice (*N* = 6, each group). E Peak increase in nerve activity following addition of 0.1mM, 0.3mM, 1mM & 3mM adenosine above baseline activity in Na_v1.9 +/+ and Na_v1.9 -/- mice (*N* = 6, each group; *p* < 0.001, 2-way ANOVA with Bonferroni's post-hoc, **p* < 0.05, ***p* < 0.01, ****p* < 0.001).

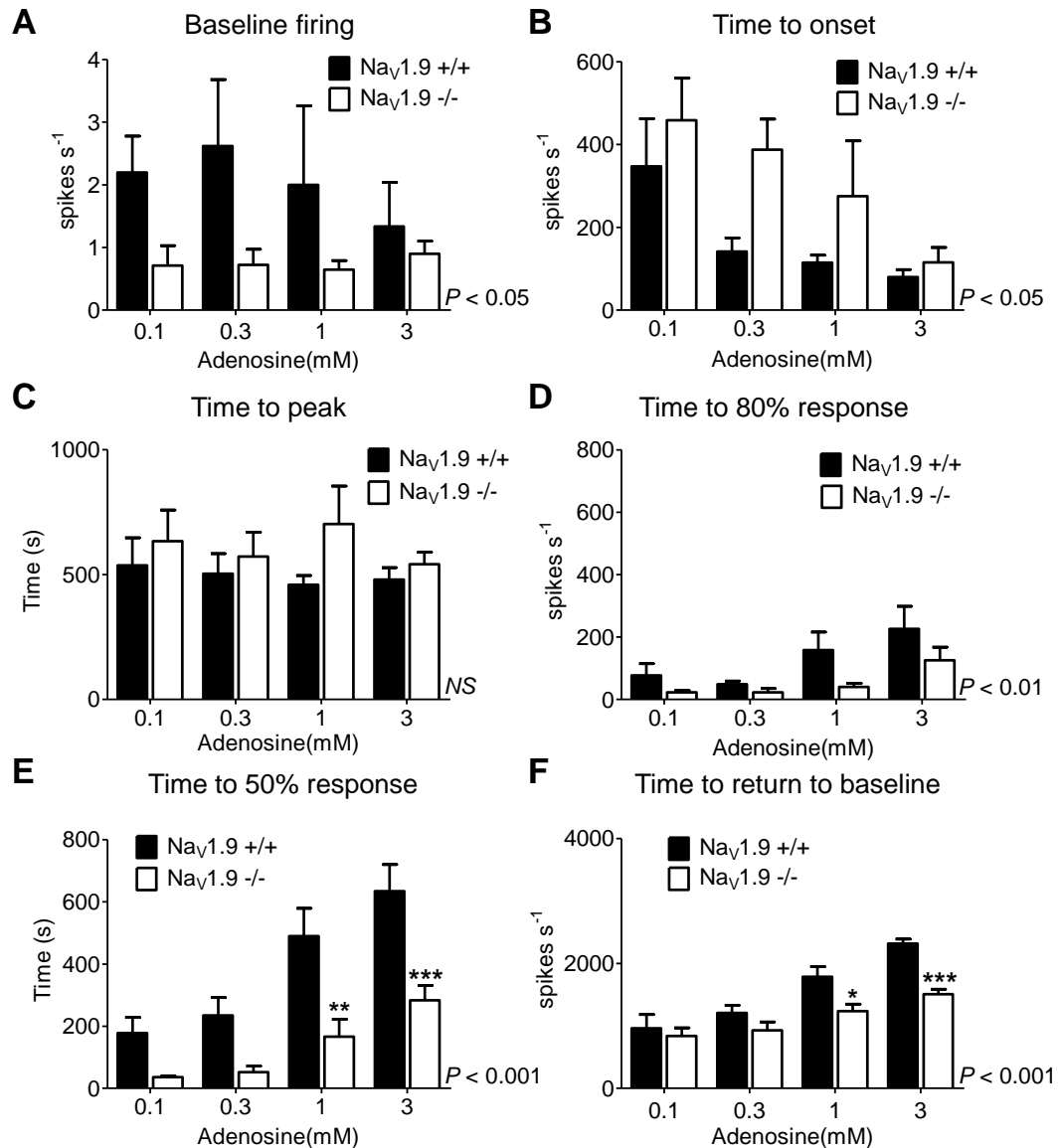


Figure 44. Baseline firing prior to adenosine application and duration of responses to adenosine in colonic splanchnic nerve. A Mean nerve activity over 3 minutes prior to application of adenosine in Na_V1.9 +/+ and Na_V1.9 -/- ($N = 6$, both groups; $p < 0.05$, 2-way ANOVA). B Time taken from adenosine superfusion entering the recording bath to an increase in mean nerve activity above baseline ($N = 6$, both groups; $p < 0.05$, 2-way ANOVA). C Time taken from adenosine entering the recording bath to reach peak nerve activity ($N = 6$, both groups; $p = 0.09$, 2-way ANOVA). D Time taken for nerve activity to return to 80% of peak response ($N = 6$, both groups; $p < 0.01$, 2-way ANOVA). E Time taken for nerve activity to return to 50% of peak response ($N = 6$, both groups; $p < 0.001$, 2-way ANOVA with Bonferroni's post-hoc, $**p < 0.01$, $***p < 0.001$). F Time taken for nerve activity to return to baseline ($N = 6$, both groups; $p < 0.001$, 2-way ANOVA with Bonferroni's post-hoc, $*p < 0.05$, $***p < 0.001$).

4.4.6 ACTIVATION OF HUMAN MESENTERIC NERVES OF THE APPENDIX, COLON AND ILEUM BY PURINERGIC LIGANDS

To translate murine activation of colonic visceral afferents by P2Y ligands to man, mesenteric nerve recordings were made from resected bowel tissues from patients undergoing surgery for inflammatory bowel disease and bowel cancer (> 10cm away from the cancer itself, cancer margins and lymph nodes).

Electrophysiological recordings were made from three tissue regions: ileum, colon and appendix. In all tissue types recorded, ATP elicited increases in human afferent firing above baseline firing (Figure 45). Similarly, the addition of P2Y receptor agonists, ADP and UTP evoked human afferent firing (Figure 45).

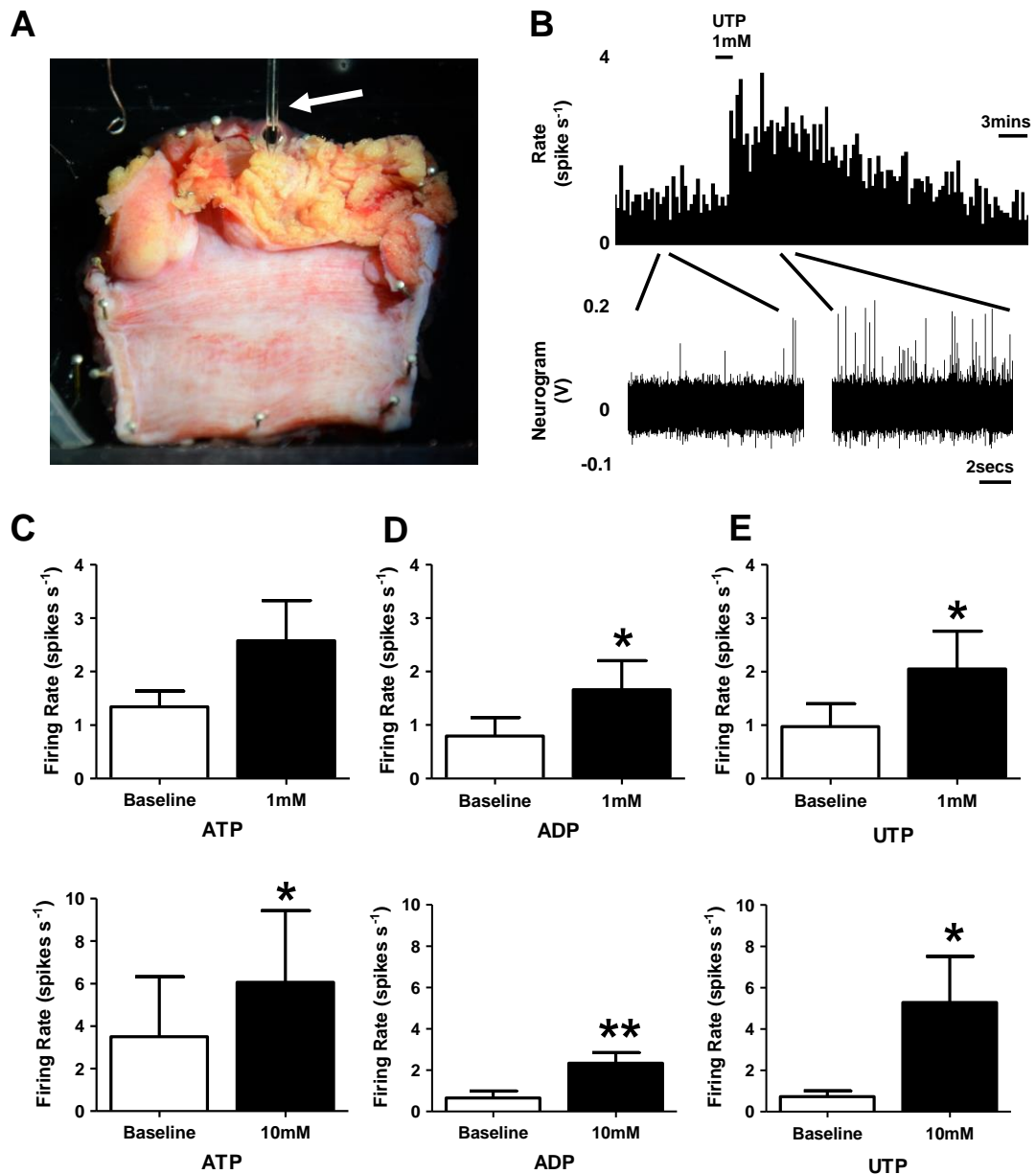


Figure 45. Effect of nucleotides, ATP, ADP and UTP, on activity of human mesenteric afferents from resected bowel tissues. A Example photomicrograph of section of ileum pinned in recording chamber serosa upwards. The mesenteric fan is at the top of the photo and a mesenteric nerve has been dissected free. The white arrow highlights the suction electrode recording from this mesenteric nerve. B Example rate histogram and raw neurogram of nerve activity following addition of 1mM UTP. Baseline firing and peak firing following addition of 1mM ($N = 3$) and 10mM ($N = 4$) ATP (C), 1mM ($N = 5$) and 10mM ($N = 6$) ADP (D) and 1mM ($N = 5$) and 10mM ($N = 4$) UTP (E). * $p < 0.05$, ** $p < 0.01$, peak firing vs baseline (paired t-test). Of the 14 independent electrophysiological recordings expressed here, 4 were made by D.C. Bulmer and 5 were made by C. McGuire. All analysis and preparation of figures was performed by J. Hockley.

Further comparison of the baseline firing rates showed no significant difference between tissue type (i.e. ileum, colon and appendix) (Figure 46). This suggests that the mesenteric nerves being dissected and recorded from were relatively consistent in size, ectopic background activity and number of nerve fibres irrespective of origin along the gastrointestinal tract. Furthermore, comparison of baseline activities prior to the addition of nucleotide agonists (ATP, ADP and UTP), show no significant trend or difference, suggesting that a nerve size bias was not a likely to explanation for the differences observed in firing frequencies between agonists.

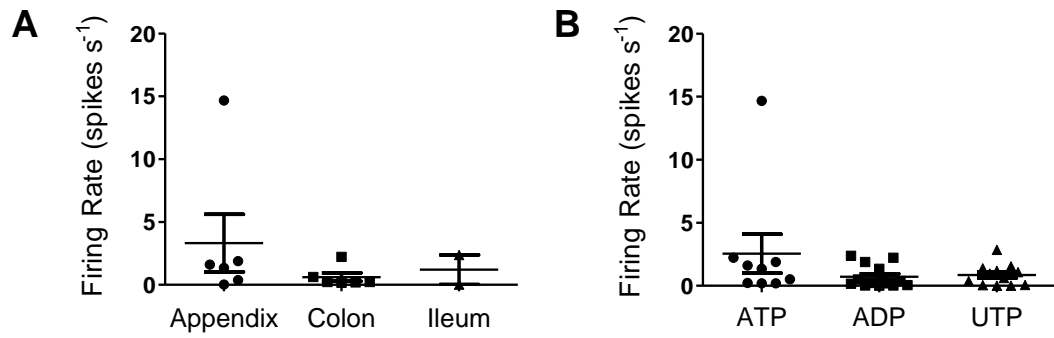


Figure 46. Comparisons of baseline firing from recordings of human mesenteric nerve activity. A Baseline firing segregated by tissue type from which the recording was made (appendix, $n = 6$; colon, $n = 6$; ileum, $n = 2$). Baseline firing rate was taken prior to the first agonist addition in that recording. B Baseline firing segregated by nucleotide agonist addition (ATP, $n = 9$; ADP, $n = 14$; UTP, $n = 12$). Baseline firing rate was taken prior to every agonist addition of each recording.

4.5 SUMMARY OF KEY FINDINGS

4.5.1 EFFECT OF $Na_v1.9$ DELETION ON COLONIC AFFERENT RESPONSES TO ATP AND ADENOSINE

1. In a tubular preparation, concentration-dependent responses to ATP and adenosine were significantly attenuated in $Na_v1.9^{-/-}$ mice compared to $Na_v1.9^{+/+}$ mice.

4.5.2 ANTAGONISM OF P2X RECEPTORS AND P1 ADENOSINE RECEPTORS ON RESPONSES TO REPEAT ATP APPLICATIONS

1. Responses to repeat applications of ATP were reduced by 22% following pre-incubation with non-selective P2X receptor antagonist, PPADS.
2. In similar experiments, ATP responses were inhibited by 18% following application of P2X₁, P2X₃ and P2X_{2/3} antagonist, TNP-ATP.

4.5.3 ACTIVATION OF COLONIC AFFERENTS BY P2Y LIGANDS

1. Application of P2Y₁, P2Y₁₂ and P2Y₁₃ receptor agonist, ADP evoked concentration-dependent increases in afferent firing of a greater magnitude than equivalent responses to ATP
2. Further, P2Y₂, P2Y₄ and P2Y₆ receptor agonist, UTP also elicited significant increases in firing, which were both shorter in duration and less in magnitude than ATP.
3. In both instances, responses to ADP and UTP were significantly reduced in $Na_v1.9^{-/-}$ mice compared to $Na_v1.9^{+/+}$ mice.
4. Application of P2Y₆ receptor agonist UDP failed to excite colonic afferent firing until reaching 10mM concentrations, where a modest increase in firing rate was observed.

4.5.4 EXPRESSION OF P2Y₁ AND $Na_v1.9$ IN COLON SENSORY NEURONES

1. P2Y₁ mRNA was observed in $26.9 \pm 5.2\%$ and $Na_v1.9$ mRNA was observed in $56.1 \pm 4.9\%$ of Fast Blue-positive colon-projecting neurones

2. Co-localisation of Nav1.9 and P2Y₁ receptors was observed in $13.7 \pm 3.8\%$ of colon-projecting neurones

4.5.5 ACTIVATION OF HUMAN COLONIC MESENTERIC NERVES BY PURINERGIC AGONISTS

1. Application of ATP, ADP and UTP all evoked concentration-dependent increases in afferent firing above baseline in human mesenteric nerve preparations from resected bowel tissues.
2. Baseline firing did not differ significantly regardless of tissue (appendix, colon and ileum) or agonist under investigation.

4.6 DISCUSSION

Data shown above further supports the role of Nav1.9 in the direct excitatory responses of colonic afferents to algogenic mediators. Nav1.9 is required for their direct excitation by multiple purinergic mediators, including ATP and adenosine. Activation of colonic afferents by P2Y receptor agonists and incomplete inhibition of ATP responses by P2X receptor, and adenosine P1 receptor antagonists indicates that the excitatory action of ATP is evoked by activation of multiple purinoceptors. Indeed, the data also shows that ATP, ADP and UTP excite human visceral afferents, suggesting, for the first time, that P2Y receptor activation of visceral peripheral nerves may be relevant to nociceptive pathways in man.

The increase in visceral afferent activity following exogenous application of ATP is significantly attenuated in the distal colon of Nav1.9 $-/-$ mice. In wild-type animals, this ATP-mediated excitation of visceral afferents was significantly reduced by PPADS and TNP-ATP (P2X receptor antagonists) and CGS 15943 (P1 adenosine receptor antagonist). Both ADP (P2Y_{1,12,13} agonist) and UTP (P2Y_{2,4} agonist) induce action potential firing, suggesting that P2Y receptor activation can excite visceral afferents. These excitatory effects to ADP and UTP were significantly reduced in Nav1.9 knock-out mice. The role of P2Y₁-dependent activation in this pathway is supported by the co-expression of P2Y₁ with Nav1.9 in a subset of colonic sensory neurones. Together, these data suggest both a prominent role for Nav1.9 in visceral afferent signalling to purinergic stimuli and also an important contribution of P2Y receptors to purinergic signalling. Given the importance of ATP mediated mechanotransduction and the

sensitisation of sensory nerves to visceral hypersensitivity, it is likely that P2Y receptors contribute significantly to the discomfort and pain experienced during inflammatory bowel disease.

4.6.1 EFFECT OF EXOGENOUS ATP ON COLONIC AFFERENT FIRING

Consistent with previous studies demonstrating the direct excitation of visceral afferents innervating the gastrointestinal tract by ATP; comparable increases in nerve discharge were observed following administration of ATP (0.1-3mM) to colonic preparations (Brierley, Carter, et al., 2005; Burnstock, 2009a; Kirkup et al., 1999; Wynn et al., 2004; Wynn et al., 2003). *In vivo* and *ex vivo* preparations require relatively high concentrations of ATP to elicit action potential firing compared to experiments on dissociated neurones. In isolated cells, the EC₅₀ of ATP at P2X₃ receptors is 1.2μM (C. C. Chen et al., 1995), with 100μM ATP triggering inward currents in most colonic neurones (Shinoda, La, Bielefeldt, & Gebhart, 2010). However, the serosal application of 100μM ATP to *ex vivo* distal colon preparations is sub-threshold for pelvic nerve activation, with concentrations closer to 1mM ATP required to evoke robust afferent firing (Wynn et al., 2003). These differences are even more marked when comparing the exogenous concentrations of ATP (~1mM) applied serosally required to excite afferent terminals, with recorded levels of ATP released intraluminally (~1-3nM) by colonic distension (Shinoda et al., 2010; Wynn et al., 2003). Even relatively high concentrations of exogenous ATP (1mM) that are known to excite colonic afferents when applied serosally, fail to consistently activate colonic pelvic afferents when applied intraluminally (Wynn et al., 2003). This data highlights the labile nature of ATP and its propensity for enzymatic

degradation by ectonucleotidases. Indeed, around 92% of ATP injected into cannulated rabbit ear arteries was not recovered in the venous outflow (HOLTON, 1959). The fact that colonic luminal perfusions of ATP did not result in consistent afferent activation is perhaps expected given the high metabolic activity of the colonic epithelium, and may also explain the low detectable levels of ATP following distension. Interestingly though, most neurones contain relatively high cytoplasmic concentrations of ATP (~2-5mM), with up to 100mM ATP observed in synaptic vesicles (Burnstock, 2009b; Novak, 2003). It is challenging to ascertain the relative amounts of ATP released by epithelial cells to shear stress. As such, it is difficult to predict the likely local concentrations of ATP exposed to colonic afferent terminals during inflammation or distension. Isolated cell experiments provide detailed information on the direct effects of ATP at purinoceptors, but lack the complexity of tissue-based assays to investigate this multifaceted neurotransmitter. By contrast, *ex vivo* preparations are likely grossly insensitive to physiological concentrations of ATP due to their rapid enzymatic breakdown, but do provide a systems approach capable of evaluating both the effects of ATP and the products of its degradation. It should be noted that both models may be biased towards the relative importance of different purinoceptor subtypes. The highly regulated breakdown and utility of ATP underlines its importance as a neurotransmitter by numerous cell types throughout the gastrointestinal tract.

4.6.2 DELETION OF Nav1.9 ON COLONIC AFFERENT RESPONSES TO CHEMICAL STIMULATION BY ATP

In Nav1.9 ^{-/-} mice, responses to ATP were substantially inhibited in colonic tissue (~83% at 1mM ATP). The magnitude of these effects would suggest that Nav1.9 is expressed by the majority of ATP-sensitive afferent fibres. Certainly in whole DRG neuronal populations, almost two-thirds of P2X₃-immunoreactive neurones also express Nav1.9, however whether this colocalisation differs in a colon-specific neuronal population remains to be confirmed (Amaya et al., 2006). Not all thoracolumbar colonic neurones are ATP-sensitive, with approximately half of DRG somata depolarised by ATP or α,β -meATP and approximately 40% of serosal afferent fibres responding to α,β -meATP application (Brierley, Carter, et al., 2005; Shinoda et al., 2010). Further, less than 40% of thoracolumbar colonic DRG neurones express P2X₃ (Brierley, Carter, et al., 2005). As such, Nav1.9 expression may only need to colocalise to less than 40-50% of colon-projecting neurones to impact the vast majority of ATP-sensitive fibres. Given the known expression pattern for Nav1.9 in colonic sensory neurones from Chapter 2, this is a realistic proposition.

4.6.3 ROLE OF SELECTIVE PURINOCEPTORS IN COLONIC AFFERENT ACTIVATION BY ATP

In order to explore the potential impact of P2Y receptor afferent activation, the response to ATP in the presence of either P2X receptor or P1 (adenosine) receptor blockade was first examined. In this study, the P2X receptor antagonists, PPADS and TNP-ATP, reduced afferent firing in response to ATP by 22% and 18%, respectively. This is in agreement with a similar reduction in the

response observed in P2X_{2/3} dual knock-out mice studies following application of ATP or α,β -meATP (Rong et al., 2009). Furthermore, given that adenosine is an ectonucleotidase break-down product of ATP and can directly excite afferents, we show that the general adenosine P1 receptor antagonist, CGS 15943, inhibited afferent discharge to ATP by 28%. This is consistent with previous reports demonstrating a similar reduction in the presence of the general P1 antagonist, 8p-SPT (Kirkup et al., 1998; Wynn et al., 2003). These findings suggest that ATP may be synergistically activating colonic afferents via P2X- and P1-dependent pathways, and that alternative purinoceptors, such as P2Y receptors, may also contribute to this excitation.

As described above, the activation of specific P2Y receptors (namely P2Y₁, P2Y₂ and P2Y₄) is excitatory in sensory neurones inducing both Ca²⁺ flux and action potential firing (Ceruti et al., 2008; Molliver et al., 2002). The P2Y₁ receptor subtype has been shown to transduce ATP-dependent mechanical stimuli and is required for thermal hypersensitivity to pain (Molliver et al., 2011; Nakamura & Strittmatter, 1996). In skin-saphenous nerve recordings, the P2Y₂ and P2Y₄ receptor agonist, UTP excites nociceptor-like fibres and more recently, the P2Y₂ receptor has been associated with the facilitation of bladder sensory function (X. Chen et al., 2010; Stucky et al., 2004). This functional activity is consistent with the expression of P2Y₁, P2Y₂ and P2Y₄ mRNA transcripts in sensory neurones (Kobayashi et al., 2006; Moriyama et al., 2003; Sanada et al., 2002; Tominaga et al., 2001). Using chromogenic *in situ* hybridisation, P2Y₁ mRNA expression was confirmed in sensory neurones and quantified in just over a quarter of colon-projecting neurones. Of these, approximately 51% co-

expressed Nav1.9 mRNA transcripts. It is perhaps surprising that, given the magnitude of the response observed to ADP application, greater expression of P2Y₁ was not seen. This may be explained by the synergistic actions of multiple purinoceptors acting in concert to excite visceral afferents. It is possible that ADP and its subsequent break-down product, adenosine, are activating multiple P2Y receptors (P2Y_{1,11-13}) and P1 receptors (A₁, A_{2A}, A_{2B} and A₃). Indeed, this can be inferred from the extended time course profiles of whole-nerve responses to adenosine, ADP and ATP compared to UTP (Figure 41), which suggests that approximately 50% of the ADP response in multi-unit recordings may be attributable to adenosine receptor activation. As mentioned above, a 28% reduction in peak response to ATP following inhibition with the P1 antagonist, CGS 15943, also implicates a significant contribution of adenosine receptor activation to ATP and ADP responses. This may, therefore, account for the disparity between the relatively low expression of P2Y₁ in colonic neurones and afferent responses to ADP. Whilst direct evidence does not exist for other P2Y receptor expression in colon specific neuronal populations, expression of P2Y₂ and P2Y₄ receptors have been shown in DRG neurones projecting to other visceral organs (X. Chen et al., 2010; H. Wang et al., 2010). Data presented above demonstrates the ability of the P2Y receptor agonists, ADP (P2Y₁, P2Y₁₂ and P2Y₁₃) and UTP (P2Y₂, P2Y₄ and P2Y₆) to excite colonic visceral afferents. It is likely that these GPCRs are facilitating excitatory responses in afferents by interactions with multiple channels, including P2X₂ and P2X₃ receptors and Na⁺ and K⁺ channel subtypes.

In Nav1.9 -/- mice, responses to ADP and UTP were significantly reduced by between 60-80%. This is suggestive of the significant expression of Nav1.9 on afferent fibres responsive to P2Y agonists. These findings are supported by the inability to develop mechanical hypersensitivity in Nav1.9 -/- mice following intraplantar injection of UTP (Amaya et al., 2006), which suggests that sensitisation evoked by UTP is significantly dependent on Nav1.9. Indeed, in sensory neurones labelled with Fast Blue from the colon, half of P2Y₁+ neurones co-expressed Nav1.9 mRNA. The expression profile of other purinoceptors (including P2Y₂ and adenosine P1 receptors) in relation to Nav1.9 is currently unknown. It is unlikely that the activation of P2Y₁, and its coupling to Nav1.9, is solely responsible for the reductions in responses to ATP and ADP observed in Nav1.9 -/- mice. As hypothesised by Baker *et al.*, increased neuronal excitability dependent on Nav1.9 current potentiation by ATP will contribute to any change in afferent firing rate, in conjunction with established contributions of Nav1.9 to both the resting excitability and sensitisation of sensory neurones (Baker, 2005).

The peak response observed to ADP was greater than that of UTP in mouse colonic splanchnic recordings. This may be due to the differential expression of P2Y receptor subtypes, alongside the synergistic activation of P2Y and P1 receptors, following the break-down of ADP, through AMP, to adenosine. This synergy in metabolites is unlikely to occur for UTP, where application of the break-down product and P2Y₆ receptor agonist, UDP, produced no excitation of visceral afferents at concentrations other than at 10mM. It is likely, therefore, that P2Y₆ receptors do not play a role in direct visceral afferent fibre activation.

Furthermore, this would also suggest that UTP, which has limited affinity to P2Y₆ receptors, is not acting via this mechanism to stimulate colonic afferents in the present study. Indeed, this is supported by the limited evidence for expression of P2Y₆ receptors in sensory neurones (Kobayashi et al., 2006). Finally, it was shown that both ADP and UTP, in addition to ATP, are able to stimulate visceral afferents innervating isolated human gut, indicating that P2Y receptors are likely to be important in sensitising afferent fibres in man. In contrast to findings in mouse studies, peak responses in human recordings were greater for UTP than ADP. Possible explanations for this include a switch in the relative expression of P2Y₂ versus P2Y₁ receptors in man, compared to mouse. Analysis of baseline firing rates dependent on tissue type (appendix, colon or ileum) and nucleotide addition (ATP, ADP and UTP), suggest no bias or significant difference in the quality or afferent population size recorded from. Teased-fibre recordings of characterised afferent fibre subtype (i.e. mesenteric, muscular, mucosal and serosal) responses to ADP and UTP in both mouse and man will provide evidence for any change in relative expression between the species, or in any bias in the populations of the afferent subtypes being stimulated.

4.6.4 CONCLUSIONS

In summary, the data demonstrates that ATP signalling is dependent on the presence of Nav1.9 in activation of colonic splanchnic afferents by ATP. The findings suggest that in addition to reduced afferent excitability driven by the loss of Nav1.9, a P2Y-Nav1.9 coupled pathway may contribute to afferent excitation and that P2Y receptor activation is important in its own right,

including the translation of P2Y activation into human nerves (Figure 47). Given the importance of ATP as a mediator of visceral pain in pathologies characterised by both inflammation and mechanical hypersensitivity, the data suggests that a mechanistic link between Nav1.9 and P2Y receptors should be investigated in more detail.

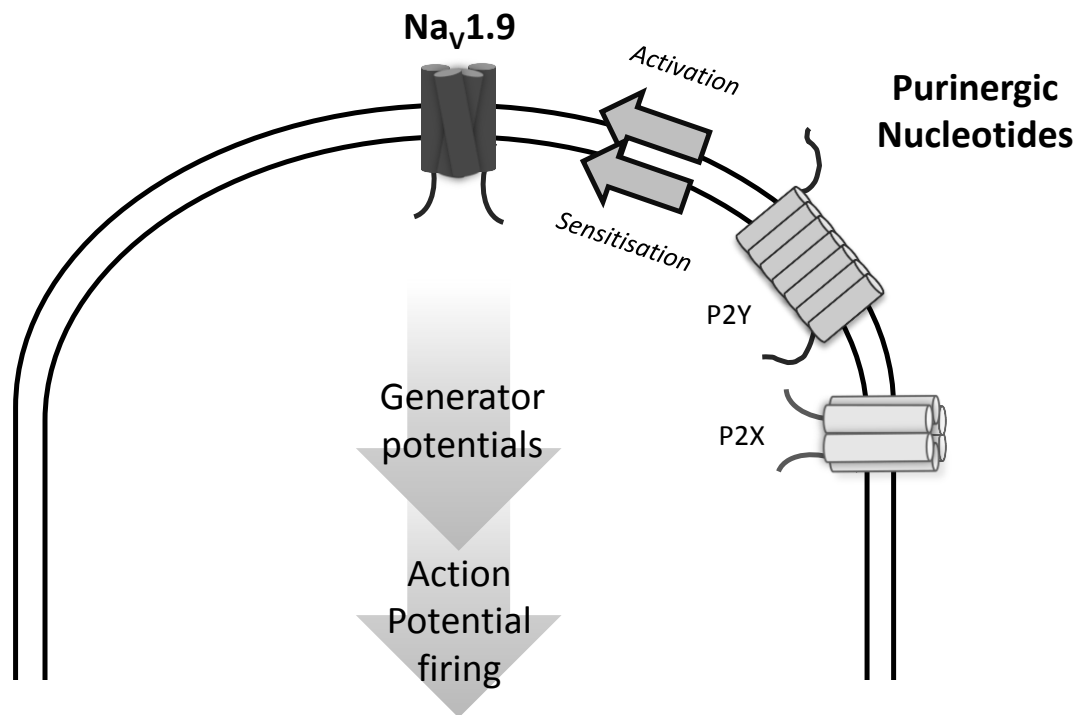


Figure 47. Schematic illustration of $\text{Na}_v1.9$ interacting with P2Y receptors in a visceral afferent terminal. When present at the visceral afferent terminal, $\text{Na}_v1.9$ may sensitise visceral afferents by interactions with P2Y receptors, possibly by potentiation of the peak current density produced by the Na^+ channel. Purinergeric nucleotides will also sensitise visceral afferent terminals by activity at P2X receptors.

CHAPTER 5: DISCUSSION

The data presented here demonstrates that Nav1.9 is a key modulator of neuronal excitability and sensitivity in colonic afferents responsive to noxious stimuli. Nav1.9 plays an important role in the regulation of visceral afferent firing to direct noxious mechanical stimuli and is essential for excitatory responses to inflammatory mediators released during acute and chronic inflammation. Specifically, we show that Nav1.9 contributes to the mechanosensitivity of serosal and mesenteric nociceptors, particularly during persistent (repeat phasic distensions) or sustained (ramp distension) noxious distension of the colon. Nav1.9 is necessary for the direct activation of visceral nociceptors by mediators released during human inflammatory disease and mechanical hypersensitivity induced by inflammatory mediators.

5.1 SUMMARY OF RESULTS

Visceral nociceptors are responsive to a range of noxious mechanical and chemical stimuli evoked during human disease (Su & Gebhart, 1998). For example, in inflammatory bowel disease, recurrent inflammation leads to abdominal pain and visceral hypersensitivity associated with physiological bowel movements. As such, the identification of points of convergence in signalling pathways common to the activation of visceral afferents by both mechanical and inflammatory stimuli will represent attractive drug targets. Such regulators of neuronal excitability will likely be capable of modulating the transduction of multiple stimuli evoked by on-going inflammation and release of inflammatory mediators. Voltage-gated sodium channels offer such a convergence point due to their intrinsic involvement in action potential generation. Of the sodium channel subtypes, Nav1.9 represents an important

molecular candidate capable of fulfilling this role. Indeed, the regulation of visceral afferents excitability by Nav1.9 has not been extensively investigated. The data presented here demonstrates that Nav1.9 represents an important modulator of specific aspects of sustained visceral afferent excitability, especially in responses to inflammatory mediators, indicating that Nav1.9 represents a valuable therapeutic target in the development of visceral analgesics.

Using a combination of *in situ* hybridisation and immunohistochemistry, Nav1.9 is present in just over half of colon-projecting sensory neurones. This expression profile indicates functional specialisation of afferents projecting to the colon and suggests that Nav1.9 may have a greater impact in specific afferent subtypes.

Next the impact of Nav1.9 on mechanosensation was investigated. Multiple mechanical stimuli were used to characterise specific afferent subtypes and examine mechanosensitivity under different dynamic and persistent paradigms. These stimuli included von Frey hair probing and circumferential stretch of isolated visceral nociceptor receptive fields, alongside repeated phasic distension of the colon at noxious pressures, and ramp distensions of tubular *ex vivo* colon preparations into noxious distension ranges. Such studies of visceral afferent mechanosensitivity have been used extensively to further our understanding of mechanosensory physiology (Feng, La, Schwartz, et al., 2012) and the molecular targets responsible for mechanotransduction in visceral afferents (Brierley et al., 2008; Page, Brierley, et al., 2005). The data presented in this thesis shows that Nav1.9 contributes to both activation thresholds for

mechanical stimuli in a subset of afferents and is required for the development of mechanical hypersensitivity to inflammatory mediators. Specifically, mechanosensory responses of mesenteric, but not serosal and muscular, afferents were significantly attenuated by Nav1.9 deletion in flat-sheet preparations. These afferents fibres also exhibited increased activation thresholds compared to wild-type controls, suggesting a reduction in sensitivity. By contrast, in tubular preparations, high-intensity stimuli including initial rapid phasic distension and supra-physiological ramp distension pressures are largely unaffected by loss of Nav1.9. Further, high-intensity von Frey hair probe of mesenteric afferent fibres did not differ significantly between genotypes, indicating that given a strong enough stimuli, it is possible to overcome the absence of Nav1.9 depolarisations in these fibres. These findings also highlight the importance of the dynamic nature of the stimulus in the activation of visceral afferents. The reduction in sensitivity of mesenteric afferents to low-intensity stimulation likely accounts for the significant deficits observed in whole-nerve recordings during tubular distension paradigms. Further, the role Nav1.9 plays in regulating responses to persistent stimuli will also impact these recordings, with Nav1.9 contributing significantly to mechanosensory responses following repeat phasic distensions.

Typically in wild-type animals, repeat colorectal distension leads to an initial desensitisation of responses that subsequently stabilises over time (Kamp et al., 2003; Sivarao et al., 2007). Interestingly in Nav1.9 ^{-/-} mice, these responses continue to decline with subsequent distension, and therefore show that Nav1.9 is required for the maintenance of responses to persistent stimuli. On-going

adaptation to repeat stimuli contributes significantly *in vivo*, where pain is evoked by repeated contractions of the colon around a bolus or stricture, and suggests a role for Nav1.9 in the adaptive response (Brookes et al., 2013). Further, repeat innocuous distension can rapidly transition from discomfort to pain if maintained (Cervero, 1988), suggesting that mechanisms regulating this process will be of significant clinical value. The molecular correlates of this tachyphylaxis in visceral afferents are poorly understood, but may depend on the progressive desensitisation of known visceral mechanosensitive channels to repeat stimuli, such as TRPV4, TRPA1 and ASIC3, with a concurrent activity-dependent functional up-regulation of Nav1.9, resulting in the maintenance of afferent activation. In this context, Nav1.9 may be considered an important regulator of gain in the primary visceral afferent terminal.

Next purinergic signalling in visceral afferents was investigated. ATP is released from the colorectal epithelial lining following mechanical stress and leads to stimulation of visceral nociceptors, primarily mediated by the activation of P2X₃ receptors (Burnstock, 1999, 2009a). Indeed, purinergic mechanotransduction contributes to the excitation and sensitisation of sensory nerve terminals during distension (Burnstock, 1999). The release of ATP is greatly enhanced during colitis supporting a greater role for this pathway during inflammation (Calvert et al., 2008; Shinoda et al., 2010; Wynn et al., 2004). Extracellular ATP may activate both P2X and P2Y receptors, suggesting that they are both capable of contributing to altered sensation in IBD. The purinergic agonists, ATP and adenosine, both significantly excited the colonic visceral afferents of wild-type animals in the current studies. Activation of colonic afferents by P2Y receptor

agonists and only partial inhibition of ATP responses by P2X, and adenosine P1, receptor antagonists indicates a role for multiple purinoceptors in the excitatory actions of ATP. Further, the activity of ATP, ADP and UTP in recordings from resected human bowel tissues, suggests that P2Y receptor activation of visceral afferents may be relevant to human nociception. These direct excitatory responses to ADP and UTP were reduced in Nav1.9 ^{-/-} mice. The role of P2Y₁-dependent activation in this pathway is supported by co-expression of P2Y₁ with Nav1.9 in a subset of colonic sensory neurones. These data taken together suggest that Nav1.9 plays a significant role in visceral afferent signalling to purinergic stimuli and that this is facilitated by P2Y receptor signalling.

Finally, the multiple mediators and human disease-derived stimuli released during tissue damage and inflammation, which are known to activate and sensitise afferent terminals, were investigated (Su & Gebhart, 1998). In addition to ATP, prototypic inflammatory mediators which are released from damaged or stressed cells include bradykinin, PGE₂, histamine and 5-HT. These have all been shown to influence visceral afferent excitation and sensitisation, and are associated with human inflammatory disease (Grundy, 2004). The present data shows that Nav1.9 is necessary to evoke excitatory firing to an inflammatory soup consisting of a cocktail of these mediators (bradykinin, ATP, histamine, PGE₂ and 5-HT). Further the impact of a human disease-derived inflammatory milieu was investigated by application of IBD (Crohn's disease and ulcerative colitis) supernatants to mouse visceral afferent fibres. Excitatory responses to these supernatants were significantly attenuated in Nav1.9 ^{-/-} mice. The loss of

sensitivity to purinergic stimuli, experimental and human disease-derived inflammatory mediators supports a more generalised role for Nav1.9 in afferent excitability by multiple inflammatory pathways in human disease. Specifically, it suggests that Nav1.9 contributes significantly to inflammatory pain sensation and to afferent sensitivity in IBD.

Mechanical hypersensitivity of serosal and mesenteric afferents is observed after application of inflammatory soup in wild-type mice, an effect that is lost in Nav1.9 -/- mice. The response to noxious ramp distension is also sensitised by intraluminal inflammatory soup, which, at least during lower distension pressures (<50 mmHg), is entirely dependent on Nav1.9. This further supports the hypothesis that Nav1.9 is critical in the sensitisation of afferents to inflammatory stimuli. Given that mesenteric afferent subtypes comprise half of colonic splanchnic innervation and mechanosensory deficits were only observed in this population following von Frey hair probing, it is tempting to postulate that this population may be functionally representative of the Nav1.9 expression pattern (approximately 50% Nav1.9 mRNA and protein) observed by *in situ* hybridisation and immunohistochemical studies (Brierley et al., 2004). However, chemosensory responses to both experimental and human disease-derived inflammatory mediators are significantly reduced in both mesenteric and serosal afferent subtypes, suggesting that this is not the case. More likely is a complex expression pattern in a sub-population of afferents with receptive fields associated with mesenteric and intramural vasculature within the colon wall, and further structure-function studies are required to confirm this. Whether the deficits in mechanotransduction in mesenteric

receptive fields of Nav1.9 ^{-/-} mice are due to specialisation in molecular channels, including mechanosensitive channels such as TRP and ASICs (Brierley et al., 2009; Brierley et al., 2008; Page, Brierley, et al., 2005), or are due to structural differences in the transduction of mechanical forces between the mesentery and serosa, remains to be seen.

Collectively, this data supports an important physiological role for Nav1.9 in the regulation of visceral afferent sensitivity during inflammation, and one that will likely have clinical relevance to human visceral pain. The role of distinct sodium channel activity in human afferent function has been indirectly addressed here by combining human *ex vivo* samples with the use of null mutant mice. This approach enables the conclusion that Nav1.9 is an important target for the treatment of visceral pain (Figure 48).

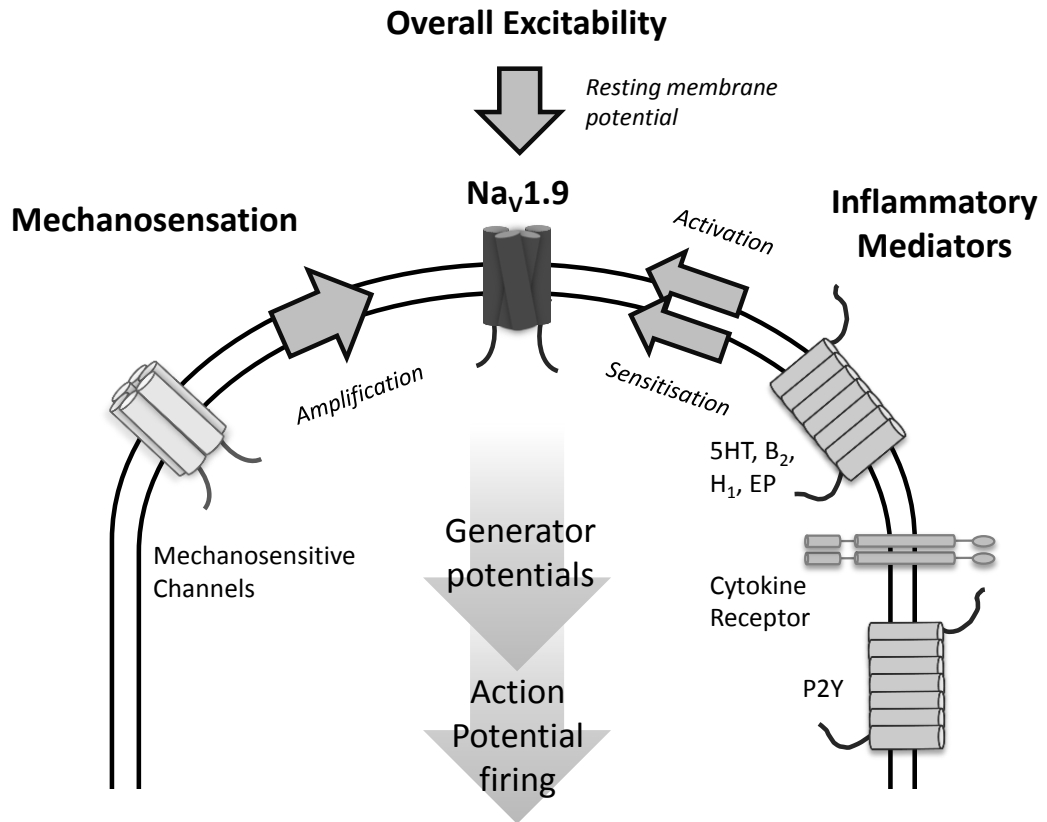


Figure 48. Schematic illustration of Na_V1.9 interacting with membrane-bound receptors and ion channels in a visceral afferent terminal. When present at the visceral afferent terminal, Na_V1.9, as well as contributing to setting the resting membrane potential, acts to amplify generator potentials evoked by mechanosensitive channels, and functions as a key transducer of inflammatory mediators and other sensitising stimuli.

5.2 THE ROLE OF Nav1.9 IN PAIN PATHWAYS

The data presented here shows that Nav1.9 acts to regulate visceral primary afferent sensitivity to multiple noxious mechanical, inflammatory and human disease-derived stimuli. The impact of altered excitability of visceral primary afferents to human pain pathways and sensation in the gut is significant. Indeed, visceral pain associated with IBD may be considered a combination of inflammatory and neuropathic pain modalities resulting from afferent nerve injury and central sensitisation, coupled to impaired descending pain modulation. Primary afferent input is likely to be critical for maintaining such chronic pain sensations (Haroutounian et al., 2014) and in some patient groups, the inhibition of visceral afferents by topical application of local anaesthetic is efficacious in treating visceral pain (Verne et al., 2003; Verne et al., 2005). As such, the modulation of viscera-projecting neuronal hyperexcitability by therapeutics targeting Nav1.9 is likely to be successful in reducing visceral pain. However, the restricted function and expression of Nav1.9 within the cell bodies and projections (central and peripheral) of sensory neurones suggests that plasticity in spinal and supraspinal contributions to a neuropathic pain component may unlikely be directly modulated by Nav1.9 (S. Dib-Hajj et al., 2002). In contrast to other sodium channels (e.g. Nav1.3), Nav1.9 expression in sensory neurones is down-regulated following axotomy and in peripheral nerve injury models, and does not contribute to neuropathic pain associated with such models (Decosterd et al., 2002; S. D. Dib-Hajj et al., 1999; S. D. Dib-Hajj et al., 1998). Whether regulation of primary afferent input by inhibition of Nav1.9 is sufficient to inhibit spinal cord dorsal horn neuronal hyperexcitability,

especially given the convergence of visceral inputs in central pathways, remains to be seen (Cervero et al., 1992). Further still, the role of Nav1.9 in the long-term chronic sensitisation of both visceral primary afferents and its contribution to ascending pain pathways is largely unknown. As such, studies in Nav1.9 null mice in chronic visceral hypersensitivity models, including both post-infectious (*Nippostrongylus brasiliensis*) and chemically sensitising protocols (trinitrobenzenesulfonic acid (TNBS)) need to be investigated. Finally, psychological factors and central sensitisation that contribute to pain thresholds in IBS-associated post-inflammatory hypersensitivity (Grover et al., 2009), are unlikely to be significantly altered by peripherally acting therapeutic agents. Therefore, dependent on the relative importance of aberrant aspects driving pain in IBD, which will itself be reliant on the degree of disease progression for an individual patient, inhibition of Nav1.9 may provide a significant mechanism to inhibit pain.

5.3 Nav1.9 CURRENTS IN COLONIC SENSORY NEURONES

The inability to record significant populations of isolated colonic DRG neurones with a Nav1.9 Na⁺ currents (present in only 0-13% of neurones) has led to the suggestion that Nav1.9 does not contribute appreciably to colonic afferent function (Beyak et al., 2004; Gold et al., 2002). However, it is likely that such reduced frequency of the observed Nav1.9 currents may be attributable to patch-clamp recording conditions and electrophysiological protocols used in isolating the persistent tetrodotoxin-resistant (TTX-R) Na⁺ current. Studies must take into account the negative activation/inactivation thresholds of Nav1.9, alongside its slow kinetics, when interrogating function. It is likely that

the -80mV holding potential used by Gold *et al.* was sufficiently depolarising to inactivate any Nav1.9 channels present and likely explains the lack of Nav1.9 Na⁺ currents observed in this study (Gold et al., 2002). A second study which found low proportions of colonic DRG neurones possessing persistent TTX-R Na⁺ currents by Beyak *et al.* utilised a holding potential (-120mV) more likely to drive Nav1.9 into a resting state, but remark that recordings were made 5 minutes after going whole-cell (Beyak et al., 2004). It has been suggested previously that due to the slow deinactivation kinetics of the channel, that cells should be held at very hyperpolarised voltages (e.g. -120mV) for up to 15 minutes to remove the inactivation block (Cummins et al., 1999). This might explain the disparity between behavioural activities and the ability to record isolated persistent TTX-R Na⁺ currents mediated by Nav1.9.

Another component of these electrophysiological recordings worth noting is the use of fluoride as the major intracellular anion in the patch-clamp pipette solution. Fluoride is used to aid seal formation during recordings. Intracellular fluoride also mimics many of the characteristics of GTP γ S, a non-hydrolysable GTP analogue, on Nav1.9 current potentiation, resulting in significantly greater current densities and a hyperpolarising shift in the voltage dependence of activation (Coste et al., 2004; Maingret et al., 2008; Maruyama et al., 2004). In fact, given that fluoride, upon binding aluminium ions, is capable of constitutively activating G-proteins, it is likely that Nav1.9 currents recorded in the presence of fluoride are significantly enhanced and do not represent endogenous current densities (Maingret et al., 2008; Matzel, Rogers, & Talk, 1996; Yatani & Brown, 1991). Nav1.9 appears to feature many unique

characteristics attributable to recording conditions. For example, unstimulated maximal current amplitude are typically in the picoAmp range, however under certain conditions may transiently potentiate to approximately 2-3 nanoAmps (Maruyama et al., 2004). Further, in the presence of intracellular GTP γ S or fluoride, this potentiation may drive Nav1.9 current amplitude up to 4-8 nanoAmps (Maingret et al., 2008; Maruyama et al., 2004; Vanoye et al., 2013). In both studies of colonic DRG neuronal populations, either chloride or methanosulphonate were used as the main anion in pipette solutions (Beyak et al., 2004; Gold et al., 2002). Whilst more physiologically relevant currents may be observed under these conditions, the introduction of fluoride into the pipette solution would have potentiated Nav1.9 Na⁺ currents over a prolonged time course and ensured that their presence in an individual colonic sensory neurone was not missed (Maingret et al., 2008; Maruyama et al., 2004). Finally, a study conducted by Hillsley *et al.* also highlights the importance of experimental design when studying channels such as Nav1.9. By utilising a 2x rheobase current injection in current-clamp recordings and recording action potential firing rates in isolated colonic sensory neurones from Nav1.9 ^{-/-} mice, the authors by-pass the proposed role for Nav1.9 in regulating resting membrane potential (RMP) and sub-threshold activation (Hillsley et al., 2006). As such, the contribution of Nav1.9 to colonic neuronal hyperexcitability is probably masked by experimental bias in the recording conditions.

Deletion of Nav1.9 will affect visceral afferent function in multiple ways. Firstly, there exists a wide 'window' current driven by the overlapping voltage dependence of activation and inactivation for Nav1.9 at, and around, the

proposed resting membrane potential for DRG neurones. As such, afferent terminals are likely to be more hyperpolarised in the absence of Nav1.9, and hence overall excitability of visceral afferents will be reduced. Secondly, Nav1.9 would no longer contribute to generator potentials through its voltage dependent activation in response to stimulation of mechanosensitive or ligand-gated ion channels. These contributions are exemplified by self-sustained plateau depolarisations and burst firing following stimulation during current-clamp recordings, which are lost in Nav1.9 -/- DRG neurones (Maingret et al., 2008). The likely consequence of these effects would be the reduced excitability and sensitivity of the afferent to stimuli. This reduction in sensitivity was observed in extracellular recordings of splanchnic nerve activity from Nav1.9 -/- mice to multiple algogenic and inflammatory stimuli (including purinergic ligands (ATP, ADP, UTP and adenosine), following application of supernatants derived from inflammatory bowel patients and after discrete application of an inflammatory soup (consisting of bradykinin, ATP, histamine, PGE₂ and 5-HT) to characterised colonic afferent receptive fields.

In addition to reductions in sensitivity caused by a loss of Nav1.9, patch-clamp studies suggest that Nav1.9 Na⁺ currents can be potentiated by inflammatory mediators acting at GPCRs (Baker, 2005; Baker et al., 2003; Rush & Waxman, 2004). Post-translational modification of Nav1.9 may therefore increase Na⁺ current density and drive significant depolarisations within the cell. In some studies, this has required the application of multiple mediators (Maingret et al., 2008), whereas in other studies, the application of a single mediator is sufficient to increase Nav1.9 currents (Baker, 2005; Rush & Waxman, 2004). In Nav1.9 -/-

mice, significantly reduced colonic afferent firing was observed to individually applied ATP, UTP, ADP and adenosine, even at supra-maximal concentrations, demonstrating that Nav1.9 is required for the activation of nerve fibres by these mediators. These findings suggest that the application of single mediators is sufficient to enhance Nav1.9 currents and trigger action potentials in visceral afferents. However, it is important to stress that other inflammatory mediators may also be present at the afferent terminal due to ongoing interactions with immune cells or gut microbiota. As a consequence, it is not possible to rule out the possibility that multiple mediators may be acting to enhance Nav1.9 currents. Another consideration is that purinergic agonists can enhance currents generated by other sodium channels (e.g. Nav1.8 (Baker, 2005)) or ion channels (Schicker et al., 2010; Tominaga et al., 2001); however, the data presented here suggest that visceral afferent excitability to these mediators is highly dependent on Nav1.9.

The enhancement of persistent Nav1.9 Na⁺ currents by ATP has been suggested to occur via G-protein coupled P2Y receptors (Baker et al., 2003). The reductions in afferent responses observed to P2Y receptor agonists, UTP and ADP, in Nav1.9 -/- mice would support a mechanistic link between these pathways. Furthermore, just over half of P2Y₁-expressing colonic DRG neurones also express mRNA transcripts for Nav1.9, suggesting that, at least in a subset of neurones, such activity may occur. It is important to stress that while loss of Nav1.9 Na⁺ current potentiation by P2Y agonists most likely contributes to the deficits observed in Nav1.9 -/- mice, the reduction in sensitivity caused by a loss of basal Na⁺ currents will also impact on neuronal excitability. This will be

particularly relevant for the subset of neurones expressing Nav1.9 but not P2Y receptors during activation with ATP. Experiments in isolated colonic DRG neurones are required to confirm the P2Y receptor subtype(s) that are responsible for any potentiation of Nav1.9 Na⁺ current in visceral afferents, which may ultimately contribute to changes in neuronal excitability.

5.4 NAV1.9 AS A THERAPEUTIC TARGET

Given the strong evidence implicating Nav1.9 in the visceral transduction of noxious inflammatory and mechanical stimuli, would a pharmacological inhibitor of Nav1.9 be an analgesic effective in the viscera? Three main points need to be addressed to begin to answer this question. Firstly, the redundancy of molecular channels capable of regulating visceral afferent sensitivity in the absence or blockade of Nav1.9. Secondly, the contribution of Nav1.9 to enteric nervous system function and thirdly, the phenotype of humans possessing Nav1.9 channelopathies need to be further understood.

5.4.1 REGULATORS OF VISCERAL AFFERENT MECHANOSENSATION AND SENSITIVITY

Multiple molecular targets regulating mechanosensation and sensitivity have been identified in recent years with preferential expression in primary afferent neurones targeting the viscera, and more specifically the colorectum. Given the significant deficits in visceral afferent sensitivity observed in Nav1.9 ^{-/-} mice, it is perhaps valuable to compare the magnitude and patterning of these phenotypes to those observed from other null mutant mice, namely TRPV1, TRPV4, TRPA1 and ASICs (Brierley et al., 2009; Brierley et al., 2008; Jones et al., 2005; Page et al., 2004; Page, Brierley, et al., 2005).

Using a combination of electrophysiological recordings of pelvic and splanchnic nerves from flat-sheet *ex vivo* colonic preparations and *in vivo* changes in behavioural responses to colorectal distension, the mechanosensory contribution of these channels has been identified. Specifically, TRPV4 and TRPA1 appear to transduce visceral mechanical stimuli relevant to nociception, with large deficits observed in serosal and mesenteric afferent subtype responses to von Frey hair probe and in behavioural responses to colorectal distension (Brierley et al., 2009; Brierley et al., 2008; Cenac et al., 2008). Responses to circumferential stretch in muscular-like afferent subtypes were unaffected by loss of either TRPV4 or TRPA1 (Brierley et al., 2009; Brierley et al., 2008). By contrast, TRPV1 null mutant mice possess significant reductions in responses to circumferential stretch of muscular-like afferents, but no difference was observed in other afferent subtypes (Jones et al., 2005). Visceromotor reflexes were also reduced in TRPV1 $-/-$ mice, suggesting that both stretch-sensitive muscular afferents and serosal/mesenteric afferents contribute to the pseudoaffective pain behaviours observed in this model (Jones et al., 2005). The role of ASICs in mechanosensation is complicated by their ability to form heteromultimers of ASIC1, ASIC2 and ASIC3 subtypes. As such, this may explain the minor increases in sensitivity of serosal afferents observed following loss of ASIC1 and ASIC2 (Page, Brierley, et al., 2005), with ASIC3 $-/-$ mice shown to have reduced responses to both serosal/mesenteric afferents and stretch-sensitive muscular-like afferent fibres (Jones et al., 2005; Page, Brierley, et al., 2005). Whilst ASIC3 knock-out affected a greater range of afferent subtypes, the magnitude of differences observed were less than those seen in TRPV4 and TRPA1 knock-outs. Though contributing to the transduction

of mechanical stimuli, ASIC3 knock-out does not alter the direct chemosensitivity of visceral afferents to inflammatory soup (Jones et al., 2005). Indeed, in visceral afferents individual knock-out of TRPV1 or TRPA1 does not impair direct excitatory responses to inflammatory soup or application of bradykinin or capsaicin, respectively, while TRPV4 mediates PAR₂-dependent excitation (Brierley et al., 2009; Brierley et al., 2008; Jones et al., 2005; Sipe et al., 2008). This suggests that whilst ASICs and TRP channels are important transducers of mechanosensitivity, their contribution to the direct activation of visceral afferents by inflammatory mediators is less well understood (with the exception to known couplings, such as TRPV4/PAR₂). What is clear is that TRPV1 and TRPA1, but not ASIC3, mediate mechanical hypersensitivity in subsets of afferent fibres (Brierley et al., 2009; Jones et al., 2005; Page, Brierley, et al., 2005). By comparison, the data presented here shows that Nav1.9 regulates direct excitatory responses to a diverse range of inflammatory mediators, mediates the subsequent mechanical hypersensitivity and contributes to aspects of mechanosensation, especially to persistent stimuli. Collectively, the loss of mechanical hypersensitivity, but not direct chemosensitivity in TRPV1 and TRPA1 knock-out mice further supports their roles as principal mechanotransducers in visceral afferent terminals, with post-translational modification of these channels likely driving an increased sensitivity to mechanical stimuli (Table 11). The impact on both direct excitation and mechanical hypersensitivity in Nav1.9 null mutant mice indicates a mechanistic role up-stream from, and integrating, mechanotransduction and chemosensitivity.

	Nav1.9	TRPV1	TRPV4	TRPA1	ASIC3	ASIC2	ASIC1
<i>Mechanosensory responses</i>							
Splanchnic mesenteric afferents	↓↓		↓↓↓	↓↓↓	↓↓	↔	↑
Splanchnic serosal afferents	↔		↓↓↓	↓↓↓	↓↓	↑	↑
Splanchnic muscular afferents	↔						
Splanchnic mucosal afferents							
Pelvic serosal afferents		↔	↓↓↓	↓↓↓	↔		
Pelvic muscular afferents		↔	↔	↓	↔		
Pelvic muscular/mucosal afferents		↓↓	↔	↔	↓↓		
Pelvic mucosal afferents		↔	↔	↔	↔		
<i>Chemosensory responses</i>							
Direct excitation	↓↓↓(IS/ATP/PGE ₂)	↔(IS)	↓↓(PAR ₂ ag.)	↔(BK/Cap)	↔(IS)		
Mechanical hypersensitivity	↓↓↓	↓		↓↓↓	↔		
Visceromotor response	↔(basal) ↓↓↓(sensitised)	↓↓	↓↓↓	↓↓	↓↓		
References	(Hockley et al., 2014; Martinez & Melgar, 2008)	(Jones et al., 2005)	(Brierley et al., 2008; Cenac et al., 2008; Sipe et al., 2008)	(Brierley et al., 2009)	(Jones et al., 2005; Page, Brierley, et al., 2005)	(Page, Brierley, et al., 2005)	(Page, Brierley, et al., 2005)

Table 11. Visceral afferent mechanosensation and chemosensation in null mutant mice. The table summarises the effects of ion channel loss on spinal afferent mechanosensory and chemosensory responses, and on behavioural responses to colorectal distension. Arrows indicate the degree of change, with blank cells indicating that this functional class of afferent or response has not been studied in that null mutant mouse. Note that whilst basal visceromotor responses are unchanged in Nav1.9 ^{-/-}, inflammatory visceral hypersensitivity is lost. IS, inflammatory soup (refer to reference for constituents); PGE₂, prostaglandin E₂; PAR₂ ag., PAR₂ receptor agonist (SLIGR); BK, bradykinin; Cap, capsaicin.

In addition to ion channels associated with the transduction of mechanical stimuli, the contribution of other known regulators of neuronal excitability, including T-type calcium channels, Kv7 and HCN2, have yet to be examined in detail in visceral afferents (Emery et al., 2011; Hirano et al., 2007; Sekiguchi & Kawabata, 2013). Importantly, the unique biophysical characteristics of Nav1.9, which produce a persistent Na⁺ current with slow activation/inactivation kinetics and hyperpolarised activation threshold, cannot be replicated by any other voltage-gated sodium channel (Catterall, Goldin, & Waxman, 2005). As such, the genetic deletion or pharmacological inhibition of Nav1.9 could not be compensated for by another Nav channel subtype. Indeed, Nav mRNA levels are largely unaffected by Nav1.9 deletion in mice (Amaya et al., 2006; Priest et al., 2005) and transient intrathecal antisense knock-down of Nav1.9 in rat produced comparable inflammatory pain phenotypes to those observed in null mutant mice, suggesting developmental up-regulation of other Navs is unlikely to explain these effects (Lolignier et al., 2011). Furthermore, normosensation in both somatic behavioural pain models and visceral colorectal distension models are unaffected by deletion of Nav1.9 (Amaya et al., 2006; Martinez & Melgar, 2008; Priest et al., 2005).

5.4.2 ROLE OF Nav1.9 IN ENTERIC NERVOUS SYSTEM FUNCTION

Considerable efforts were taken to ensure that changes observed in afferent activation in this study were not confounded by the loss of Nav1.9 within intrinsic primary afferents neurones (IPANs) of the enteric nervous system (ENS), which are known to express Nav1.9 (Padilla et al., 2007; Rugiero et al., 2003). Although the contribution of second order enteric neurones projecting

out of the gut (intestino-fugal fibres) in these recordings cannot be ruled out, these would proportionally represent a small minority of the total splanchnic outflow at the recording site central to the inferior mesenteric ganglia (Luckensmeyer & Keast, 1995). It is also currently unknown whether intestino-fugal fibres express Nav1.9. Similarly, the contribution of rectospinal neuronal projections to the present multi-unit recordings cannot be excluded. It should be said that these fibres are low in frequency and thus far have only been associated with anatomical projections through dorsal roots L6/S1: spinal segments through which the lumbar splanchnic nerve is known not to project (Neuhuber et al., 1993). Potential impact of the loss of Nav1.9 from IPANs on local reflexes was controlled by the inhibition of intrinsic smooth muscle tone by inclusion of both nifedipine (L-type calcium channel blocker) and atropine (muscarinic acetylcholine antagonist) to the perfusion buffer. These methodological steps, in conjunction with both the magnitude of the effects observed and the paucity of evidence for cross-talk between the ENS and extrinsic afferents mediating extrinsic afferent sensitivity, would strongly suggest that the changes we observe are due to the modulation of neuronal excitability by Nav1.9 in visceral extrinsic afferents (Mueller et al., 2009). Indeed, at best, intrinsic neurones are only likely to influence the small proportion (10%) of extrinsic afferents terminating at myenteric plexi between colonic muscle layers (Brierley et al., 2004; Mueller et al., 2009) and are unlikely to influence the function of vascular afferents that comprise the significant proportion of spinal afferent fibres projecting to the colorectum.

Loss of Nav1.9 from the myenteric plexus has been shown to alter spontaneous contractile activities in the mouse colon, which will impact the propulsion of digestive content (Copel, Clerc, Osorio, Delmas, & Mazet, 2013). Specifically, the frequency of colonic migrating motor complexes (CMMCs) and intensity of contraction was increased in Nav1.9 $-/-$ mice. As such, targeting Nav1.9 by pharmacological inhibitors may, in fact, facilitate colonic transit through synergistic action at both visceral afferent fibres and enteric neurones. This could be of considerable benefit to inflammatory bowel disease patients where existing efficacious visceral analgesics, namely opioids and anti-spasmodics, are typically associated with stasis and constipation (Ford et al., 2008; Mowat et al., 2011).

5.4.3 HUMAN NAV1.9 CHANNELOPATHIES AND PATHOPHYSIOLOGY

The recent identification of human Nav1.9 channelopathies associated with congenital insensitivity to pain, episodic pain syndrome and painful neuropathy has facilitated the functional study of this channel in human sensation. Familial episodic pain has been attributed to different point mutations in Nav1.9 in two Chinese families (Zhang et al., 2013). Two separate mutations at p.Ala808Gly and p.Arg225Cys caused episodic pain predominantly in the lower distal extremities, which was worsened by fatigue. These mutations, in contrast to p.Leu811Pro, cause a significant increase in current density and leave the voltage dependence of activation and inactivation unchanged (Zhang et al., 2013). This leads to significant increases in neuronal excitability, which was not attributed to changes in resting membrane potential or action potential threshold. Instead, increased action potential firing following current injections

is likely causal to the observed hyperexcitability of DRG neurones, which manifests as relapsing episodic pain occurring at a frequency of 2-5 days.

Recently eight heterozygous variants of *SCN11A* were identified in 12 patients from a cohort of 393 patients with painful neuropathy (Huang et al., 2014). Mutations in *SCN9A* and *SCN10A* were not causal and detailed functional electrophysiological analysis was carried out on two mutations (p.Ile381Thr and p.Leu1158Pro) possessed by four patients, which were shown to confer gain-of-function characteristics to the Nav1.9 channel. Symptoms presented late in life (>50 years of age) and consisted of numbness, tingling and typically dull burning pain in the lower limbs. These were associated with autonomic changes including diarrhoea, hyperhidrosis, dry mouth/eyes and altered blood flow. Specifically, p.Ile381Thr and p.Leu1158Pro mutations were located in membrane-spanning segments lining the pore (DI/S6) and in the voltage-sensor (DIII/S3) of the channel, respectively. Both mutations lead to hyperpolarising shifts in the voltage dependence of activation and, in the case of p.Ile381Thr, a depolarising shift in the voltage dependence of inactivation; whilst this remained unchanged for p.Leu1158Pro. Resting membrane potential was depolarised and action potential current threshold was reduced by both mutations, resulting in significant increases in action potential firing during depolarising current steps. Furthermore, controlling for recombinant expression levels of mutant Nav1.9 in transfected neurones using dynamic clamp, similar effects on RMP and AP firing hyperexcitability were observed.

A single *de novo* p.Leu811Pro mutation located at the distal end of the S6 transmembrane helix in domain II of Nav1.9 has been linked to the inability to

experience pain in humans (Leipold et al., 2013). Clinically, this results in multiple painless fractures and slow wound healing. Further, gastrointestinal function is also impaired with patients requiring temporary parenteral nutrition and possessing morphologically abnormal small intestine and enlarged colon (Leipold et al., 2013). This phenotype is driven by changes in Nav1.9 voltage-dependant gate closure and channel inactivation caused by the mutation. As such, Nav1.9 p.L811P possesses a leftward shift in activation and deactivation kinetics ($\sim -29\text{mV}$), and results in increased Nav1.9 Na^+ current flux at rest, and a subsequent $\sim 7\text{mV}$ depolarisation of the resting membrane potential. Leipold *et al.* hypothesise that other voltage-gated sodium channels and voltage-gated calcium channels are therefore progressively inactivated and the sensory neurone experiences conduction block (Herzog et al., 2001; Leipold et al., 2013). Given the extensive expression of Nav1.9 within small DRG neurones, this could result in a selective blockade of primary nociceptive pathways and an inability to sense pain. This hypothesis had been challenged by recent studies linking mutations in Nav1.9 with hyperexcitability of DRG neurones. Whilst the change in the voltage dependence of activation and deactivation associated with the p.L811P mutation are far greater than those seen in the Huang *et al.* and Zhang *et al.* mutations, the subsequent depolarisation in RMP observed by Leipold *et al.* of $\sim 7\text{mV}$ is comparable to that seen by Huang *et al.* (Huang et al., 2014; Leipold et al., 2013; Zhang et al., 2013). As such it is difficult to reconcile these findings with current hypotheses correlating point mutations in both Nav1.7 and Nav1.9 to depolarising changes in RMP and the subsequent *hyperexcitability* of DRG neurones (Harty et al., 2006; Huang et al., 2014). Indeed, Huang *et al.* go on to state that

'hypoexcitability of mouse DRG neurones that express the Nav1.9 L811P mutation cannot be explained by the shift in RMP of these neurones' (Huang et al., 2014). This is probably due to the expression of Nav1.8 in the vast majority of small DRG neurones, which unlike other voltage gated sodium channels, has voltage-dependencies of activation and inactivation 20-40mV more depolarised (Catterall et al., 2005). As such a 5-7mV depolarising shift in RMP is unlikely to drive significant proportions of Nav1.8 channels into inactivated states and will result in hyperexcitability of these neurones (D. L. Bennett, 2014; Huang et al., 2014), however in those cells not expressing Nav1.8, hypoexcitability is the likely phenotype (Rush et al., 2006). Further, Huang *et al.* suggest that the hypoexcitability observed in DRG neurones expressing the Nav1.9 p.L811P mutation may potentially be explained by a sampling bias towards these cells, with cells expressing Nav1.8 significantly fatigued or subnormal (Huang et al., 2014). Fluorescent dye-filled *in vivo* intracellular recordings of mouse DRG neurones possessing the Nav1.9 L811P mutation, with subsequent post-functional expression labelling for Nav1.8 would help to answer this hypothesis.

Interestingly, Nav1.5 and Nav1.9 have recently been shown to be the two sodium channels responsible for carrying the TTX-R Na⁺ currents observed in myenteric neurones (Osorio, Korogod, & Delmas, 2014). As such, given the lack of Nav1.8 in these neurones, in patients presenting with Nav1.9 p.L811P mutations, it would be predicted that myenteric neurones are hypoexcitable. The GI phenotype seen in Nav1.9 p.L811P mutants, with reduced peristaltic waves, may be a result of such myenteric dysfunction (Leipold et al., 2013). Whether myenteric neurones are hyperexcitable in Nav1.9 null mutant mice

manifesting as increased frequency of migrating motor complexes remains to be seen (Copel et al., 2013).

In patients with episodic pain syndrome associated with Nav1.9 p.Ala808Gly and p.Arg225Cys mutations, the pain region was reported as feeling extremely cold and pain could be ameliorated by hot compress (Zhang et al., 2013). One possible rationale may be the co-expression of Nav1.9 by Nav1.8-positive neurones, which are known to mediate cold sensation and hence their hyperexcitability may exploit existing thermosensation pathways (Zimmermann et al., 2007). This is in stark contrast to the presentation of Nav1.7-dependent primary erythromelalgia where severe burning pain in the extremities may be relieved by ice bath or cold compress (Fischer & Waxman, 2010). It is also tempting to speculate on the transient nature of pain bouts experienced by these patients, which tend to last between 5 and 20 minutes, approximately the duration of so-called Nav1.9 'kindling' and run-down of current amplitudes during whole-cell patch-clamp experiments (Maruyama et al., 2004; Zhang et al., 2013). However, it remains to be seen whether such kindling events are merely an artefact of patch-clamp recording techniques. Finally, that the pain was significantly attenuated by paracetamol and NSAID drugs implicates inflammatory processes in its propagation and is complicit with the role of Nav1.9 described in this thesis (Zhang et al., 2013).

What do these channelopathies tell us about Nav1.9 as an analgesic target for inflammatory pain? Importantly, cognitive function and brain development appears normal in patients possessing these Nav1.9 mutations (Huang et al., 2014; Leipold et al., 2013; Zhang et al., 2013). This may have been unexpected

given the purported requirement of Nav1.9 in neurotrophin-evoked depolarisations in mammalian brain (Blum et al., 2002). Interestingly, patients with Nav1.9 p.L811P presented with delayed motor development and mild muscular weakness, although biopsies and electromyography were normal (Leipold et al., 2013). Such motor phenotypes were not explicitly mentioned for p.Ala808Gly and p.Arg225Cys mutations (Zhang et al., 2013) or p.Ile381Thr and p.Leu1158Pro mutations (Huang et al., 2014). Indeed, one patient is reported to have been a soldier previously, at least suggesting that motor or muscular deficits, if present, were not incapacitating (Zhang et al., 2013). Nav1.9 has been implicated in the development of motoneurone axons, with Nav1.9 $-/-$ mice showing marked reductions in axon growth (Subramanian et al., 2012). This axon growth is activity-dependent on voltage-gated calcium channels and suggests that aberrant p.L811P Nav1.9 Na⁺ flux may impair or retard motoneurone development in patients with this mutation (Leipold et al., 2013). However, it is clear that these patients ultimately exhibit normal motor control, suggesting that compensation at least within motoneurons for Nav1.9 deficits can occur.

The GI phenotype presented by Leipold *et al.*, alongside rodent models of enteric neuronal function, suggests that Nav1.9 likely contributes to the effective function of the GI tract, including peristaltic propulsion (Copel et al., 2013; Leipold et al., 2013; Padilla et al., 2007; Rugiero et al., 2003). In patients with familial episodic pain as a result of alterations in Nav1.9 reported no GI dysmotility (Zhang et al., 2013). Similarly, patients with painful neuropathy associated with Nav1.9 mutations did not report GI dysfunction (Huang et al.,

2014). Collectively this data would suggest that the p.L811P Nav1.9 phenotype differs from other painful phenotypes associated with Nav1.9 mutations, not only in the context of pain sensation (or lack of) but also with regards to GI function. The aberrant function of DRG and myenteric neurones expressing Nav1.9 p.L811P mutations are, therefore, not fully explained by current hypotheses and further work is required to understand this phenotype fully.

The data in this thesis, in conjunction with pain phenotypes of human mutants and our current understanding of the function of Nav1.9, suggests a significant role for the channel in the development of visceral inflammatory pain. Together these findings suggest that pharmacological blockade of Nav1.9 may prove an effective analgesic strategy in pathologies where the predominant pain is caused by acute or on-going inflammation. This may be particularly relevant for inflammatory bowel disease, where there is an unmet medical need for mechanistically novel analgesics. Indeed, given the multifactorial character of the pain in IBD, Nav1.9 blockers in conjunction with treatments for neuropathic pain (including pregabalin/gabapentin) may result in the most benefit for patients. Other relevant conditions where inflammatory or visceral pain is a principle component include acute inflammatory conditions such as appendicitis, dermatitis and tonsillitis, or chronic diseases such as pancreatitis and rheumatoid arthritis.

5.5 FURTHER WORK

A number of important studies are required to consolidate the findings presented here. Investment in the development of Nav1.9 knock-out strains using transient and tissue-specific promoters, as has been undertaken for Nav1.7 and Nav1.8, would significantly drive the field forward (Abrahamsen et al., 2008; Minett et al., 2012). Specifically, floxed *SCN11A* under the control of *Nav1.8-Cre* would enable Nav1.9 knock-out in well characterised nociceptors. This could be extended to the use of *Wnt1-Cre* and *Advillin-Cre*-expressing mice, which would select for deletion within precursors of sensory and autonomic neurones, and trigeminal and DRG neurones, respectively. Furthermore, the development of knock-in *Nav1.9-Cre* mice crossed with globally expressed floxed diphtheria toxin A (DTA)-subunit gene, would result in the death of *Nav1.9-Cre*-expressing neurones. The absence of these neurones would provide a clear opportunity to functionally characterise colon-projecting neurones and understand the extent of Nav1.9 expression in visceral afferents.

These mutant mice could also be used to investigate the role of Nav1.9 in visceral afferent function more generally, including in vascular afferent terminals of the kidney, lung and pancreas. With regards to the GI tract, characterisation of afferent function in lumbosacral pathways of Nav1.9 global null mice is also required, alongside characterisation in vagal pathways. As mentioned above, given the importance of Nav1.9 in neuronal sensitivity during inflammatory states, studies looking into the mechanical hypersensitivity to colorectal distension in rodent *in vivo* models of both post-infectious IBS and TNBS-induced colitis are necessary. Importantly, distension paradigms that

activated splanchnic, as well as pelvic, pathways should be employed to provide parity with existing *ex vivo* data. Such *in vivo* models could be combined with the use of supernatants derived from IBS and IBD tissues to provide translation to human disease.

Finally, mechanistic studies focusing on the G-protein coupled current amplitude regulation of Nav1.9 in colonic sensory neurones are required; examining the ability for inflammatory mediators, including ATP, to up-regulate Nav1.9 currents and the likely impact of this on visceral neuronal function. Importantly, understanding whether ATP-mediated P2Y activation is a significant modulator of Nav1.9 function will further the understanding of Nav1.9 in visceral afferents.

5.6 CONCLUSIONS

Visceral afferents are polymodal and require mechanisms for regulating neuronal excitation induced by a range of innocuous and noxious mechanical and inflammatory stimuli. Indeed, visceral nociceptors are prone to modulation by inflammatory mediators, and may be recruited after being previously silent (Brierley, Carter, et al., 2005; Brierley, Jones, et al., 2005; Feng & Gebhart, 2011; Feng, La, Schwartz, et al., 2012). These results suggest that Nav1.9 represents a significant mechanism responsible for regulating visceral afferent sensitivity by noxious inflammatory, mechanical and human disease-derived stimuli. In summary, the data supports an important physiological role for Nav1.9 in the regulation of visceral afferent sensitivity during inflammation and one which will likely have direct clinical relevance to human visceral pain.

CHAPTER 6: APPENDICES

6.1 APPENDIX 1 – EXPRESSION STUDIES

6.1.1 CORRELATION BETWEEN *Na_v1.9*-IMMUNOREACTIVITY AND *IB4*-BINDING

Previous studies have shown that *IB4* binding is positively correlated to *Na_v1.9* expression, however this has not been examined in the context of visceral afferents (Fang et al., 2006). If this finding is reproducible, then *IB4*-binding may prove a more versatile surrogate representative of *Na_v1.9* expression. To investigate this correlation further during expression studies performed in Chapter 2, the intensity of *Na_v1.9*-immunoreactivity was correlated with *IB4*-binding in colon-projecting (FB+) neurones and unlabelled (FB-) neurones from rat DRG. In Figure 49, positive correlations were observed with increasing relative intensity of *Na_v1.9* linked to increasing relative intensity of *IB4*. However, whilst the correlation is significant (Pearson two-tailed test, $p < 0.0001$ (FB-) and $p < 0.001$ (FB+), Figure 49), it is relatively weak (FB+, $R^2 = 0.24$), especially within FB- neurones (FB-, $R^2 = 0.10$). In the study by Fang *et al.*, *Na_v1.9* expression was strongly correlated to *IB4* binding in a very specific set of well characterised nociceptive neurones (Fang et al., 2006). It is likely that the very specific ‘nociceptive’ phenotype selected for by Fang *et al.*, and any associated *Na_v1.9*-*IB4* correlation, is lost by the lack of functional data linked to this immunohistochemical analysis.

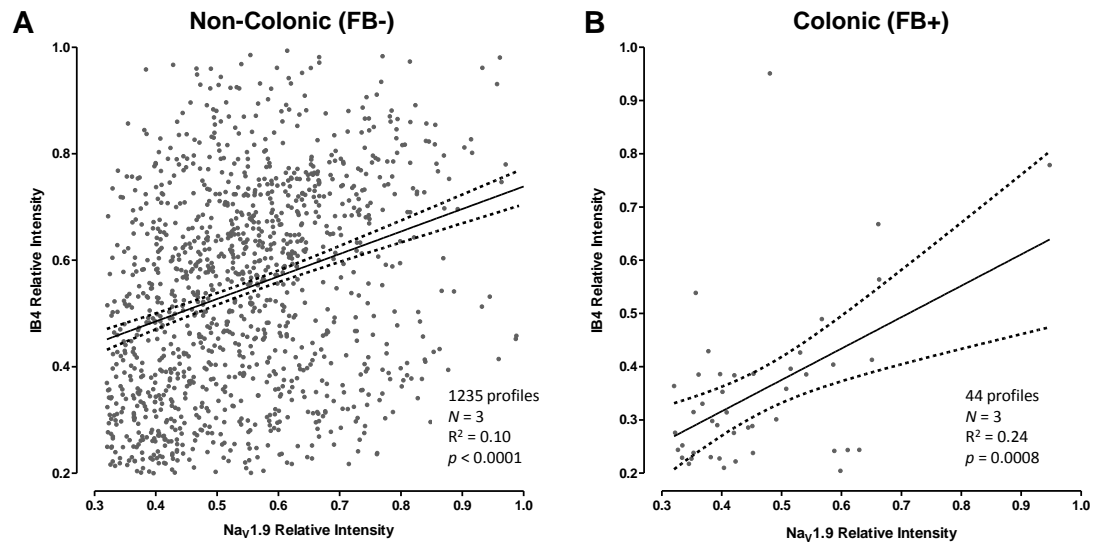


Figure 49. Correlation of relative intensity of Na_v1.9 with IB4 in colonic (FB+) and unlabelled (FB-) populations. Correlation of relative intensity of Na_v1.9+ neuronal profiles with relative intensity of IB4+ neurones profiles in FB- (A) and FB+ (B) populations. Line of best fit is shown with 95% confidence levels.

6.1.2 CORRELATION BETWEEN *Nav1.9* mRNA TRANSCRIPT EXPRESSION AND *Nav1.9* PROTEIN EXPRESSION

In a pilot study to compare the expression of *Nav1.9* mRNA transcript with *Nav1.9* protein, isotopic *in situ* hybridisation was used in conjunction with immunohistochemistry in rat DRG sections. Antisense probes previously shown to be specific for *Nav1.9* mRNA in mouse were hybridised to rat DRG sections already labelled with *Nav1.9* antibody. The probes used were specific for mouse *Nav1.9* (m*Nav1.9*) mRNA and therefore reduced binding efficiency was predicted in rat tissues (see Chapter 2 Methods (2.3.4 Isotopic *in situ* hybridisation probe selection) for *Nav1.9* species homology and probe design). In this single experiment, *Nav1.9* mRNA expression was shown to correlate with *Nav1.9* protein expression (Pearsons correlation test, $p < 0.0001$, $R^2 = 0.42$) further validating the specificity of the *Nav1.9* antibody. Whilst this study provides a methodological link between *in situ* hybridisation and immunohistochemistry datasets, this was not replicated ($N = 1$) and therefore should not be over-interpreted.

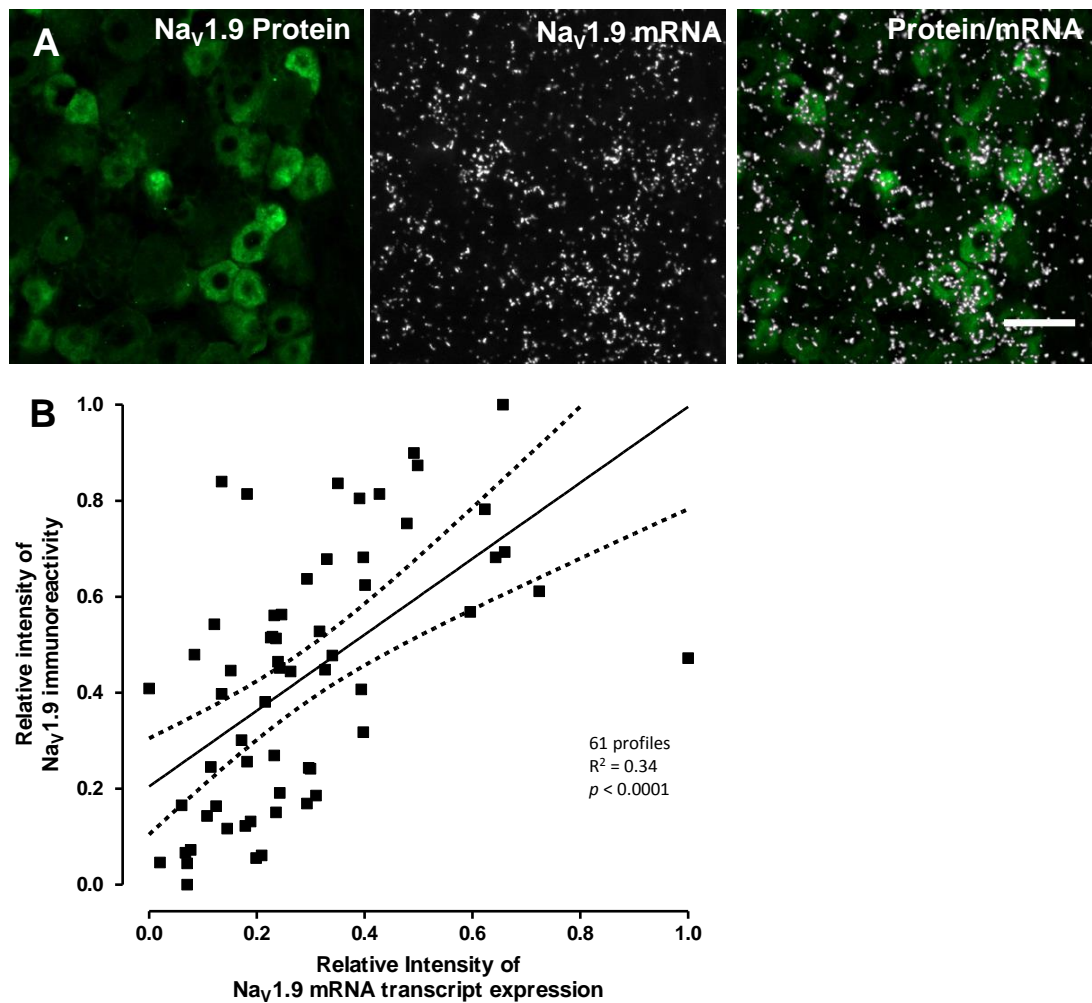


Figure 50. Colocalisation of Na_V1.9 mRNA and protein using *in situ* hybridisation and simultaneous immunohistochemistry. A Example fluorescent images showing Na_V1.9-immunoreactivity (i), Na_V1.9 mRNA expression as silver grain under polarised-light (ii) and merge (iii). Scale bar 50µm. B Scatter-plot of relative intensity of Na_V1.9 mRNA transcript expression against Na_V1.9 immunoreactivity (60 profiles, $N = 1$, $p < 0.0001$, $R^2 = 0.42$). Line of best fit (solid line) is shown with 95% confidence intervals (dashed lines).

6.2 APPENDIX 2 – FUNCTIONAL STUDIES

6.2.1 EFFECTS OF SELECTIVE P2Y RECEPTOR AGONISTS AND ANTAGONISTS ON COLONIC SPLANCHNIC NERVE ACTIVITY

In extracellular electrophysiological recordings of whole-nerve afferent activity of the splanchnic nerve in mouse, the application of P2Y receptor agonists, ADP and UTP led to significant excitatory responses. Specifically, ADP has activity at P2Y₁, P2Y₁₁, P2Y₁₂ and P2Y₁₃ receptors and UTP is specific for P2Y₂, P2Y₄ and P2Y₆ receptors. Of these, the positively G_{q,11}-protein coupled P2Y₁ and P2Y₂ receptor subtypes have been associated with many pro-excitatory effects in neuronal cells and visceral afferents (X. Chen et al., 2010; Yousuf et al., 2011). In order to investigate these effects further P2Y receptor subtype-specific agonists and antagonists were applied to whole-nerve splanchnic recordings of the distal colon in a tubular preparation (as described in Chapter 3.3.2 *In vitro* mouse colonic splanchnic afferent preparations). The concentrations used were based on usage in comparable *ex vivo* afferent nerve preparations. If this was not possible, then IC₅₀ and EC₅₀ concentrations at the receptor of interest were used to estimate an appropriate concentration to apply to colonic afferent preparations (see Chapter 4.3.8 Drugs). Wash-out of effect, reproducibility of application and concentration-dependent effects were all used to confirm specific pharmacology. Independent application of supramaximal concentrations of selective P2Y₁ agonist MRS2365 (10μM) and selective P2Y₂ agonist PSB1114 (3μM and 10μM) failed to robustly evoke afferent excitation in colonic afferent tubular preparations (Figure 51A & B). Indeed, antagonism of

P2Y₁ receptors with 3μM MRS2500 did not block either 1mM ADP responses or 1mM ATP responses (Figure 51C). Further to this, pre-incubation with selective P2Y₂ antagonist, 10μM AR-C118925X also failed to inhibit 1mM UTP responses and 1mM ATP responses (Figure 51E). Finally, potential excitatory effects of P2Y₁₂ receptor activation by 1mM ADP or 1mM ATP were discounted by pre-incubation with selective P2Y₁₂ antagonist, ticlopidine (100μM; Figure 51D). The lack of effectiveness of P2Y receptor selective ligands in this preparation, when compared to responses evoked by ADP and UTP suggests that the concentrations of, or the ligands, themselves may not be amenable to afferent nerve preparations. Given the concentrations of purines (1-10mM ATP, ADP and UTP) required to robustly excite colonic afferents it is plausible that selective P2Y receptor ligands are also restricted by equivalently active enzymatic degradation pathways. This is especially relevant given the analogous chemotypes that selective P2Y receptor ligands share with endogenous purine agonists. Alternatively, it is possible that independent selective P2Y receptor subtype activation or antagonism is inadequate to directly excite or inhibit colonic afferents.

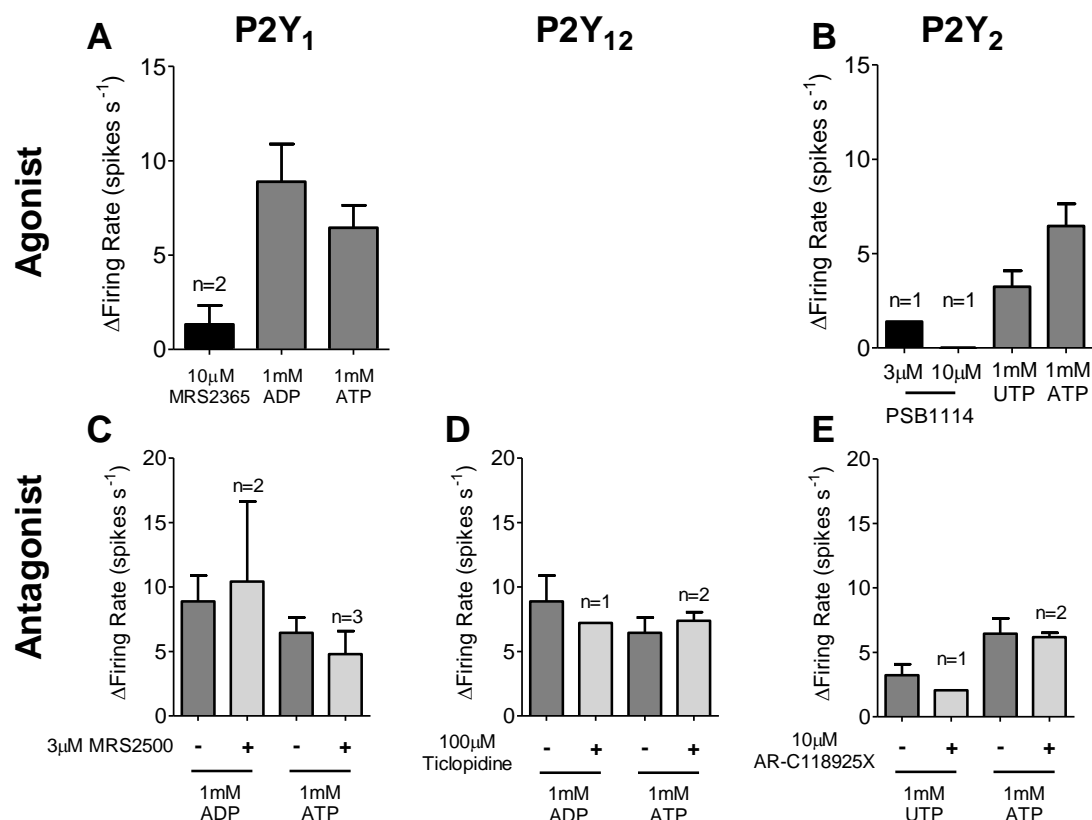


Figure 51. Application of selective P2Y receptor agonists and antagonists to colonic splanchnic nerve preparations. A Peak response to application of selective P2Y₁ receptor agonist, MRS2365 (10 μM) to colonic splanchnic nerve tubular preparation (*N* = 2). By way of comparison, 1 mM ADP (*N* = 6) and 1 mM ATP (*N* = 6) responses are also shown. B Peak response to application of selective P2Y₂ receptor agonist, PSB1114 (3 μM and 10 μM; *N* = 1 at each concentration), plotted with 1 mM UTP (*N* = 6) and 1 mM ATP (*N* = 6) responses. C Peak responses to 1 mM ADP (*N* = 2) and 1 mM ATP (*N* = 3) following pre-incubation with selective P2Y₁ antagonist, MRS2500 (3 μM) compared to 1 mM ADP and 1 mM ATP (*N* = 6) without pre-treatment. D Peak responses to 1 mM ADP (*N* = 1) and 1 mM ATP (*N* = 2) following pre-incubation with selective P2Y₁₂ antagonist, ticlopidine (100 μM) compared to 1 mM ADP and 1 mM ATP (*N* = 6) without pre-treatment. E Peak responses to 1 mM UTP (*N* = 1) and 1 mM ATP (*N* = 2) following pre-incubation with selective P2Y₂ antagonist, AR-C118925X (10 μM) compared to 1 mM ADP and 1 mM ATP (*N* = 6) without pre-treatment.

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